ACS SYMPOSIUM SERIES 1095

Small Wonders: Peptides for Disease Control

Edited by

Kanniah Rajasekaran, Jeffrey W. Cary,
Jesse M. Jaynes, and Emilio Montesinos

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.fw001

Small Wonders: Peptides for Disease Control

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.fw001

ACS SYMPOSIUM SERIES **1095**

Small Wonders: Peptides for Disease Control

Kanniah Rajasekaran, Editor
Southern Regional Research Center
U.S. Department of Agriculture
New Orleans, Louisiana

Jeffrey W. Cary, Editor
Southern Regional Research Center
U.S. Department of Agriculture
New Orleans, Louisiana

Jesse M. Jaynes, Editor
Tuskegee University, Tuskegee, Alabama

Emilio Montesinos, Editor
University of Girona, Girona, Spain

Sponsored by the
ACS Division of Agricultural and Food Chemistry, Inc.

American Chemical Society, Washington, DC

Distributed in print by Oxford University Press, Inc.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.fw001

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.fw001

Library of Congress Cataloging-in-Publication Data

Small wonders : peptides for disease control / Kanniah Rajasekaran ... [et al.], editor[s].
p. ; cm. -- (ACS symposium series ; 1095)
"Sponsored by the ACS Division of Agricultural and Food Chemistry, Inc."
Includes bibliographical references and index.
ISBN 978-0-8412-2748-4 (alk. paper)
I. Rajasekaran, K., 1952- II. American Chemical Society. Division of Agricultural and Food Chemistry. III. Series: ACS symposium series ; 1095. 0097-6156
[DNLM: 1. Antimicrobial Cationic Peptides. 2. Anti-Infective Agents. 3. Plant Diseases. 4. Therapeutics. QU 68]

615.7′92--dc23

2012004898

The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48n1984.

Copyright © 2012 American Chemical Society

Distributed in print by Oxford University Press, Inc.

All Rights Reserved. Reprographic copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Act is allowed for internal use only, provided that a per-chapter fee of $40.25 plus $0.75 per page is paid to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, USA. Republication or reproduction for sale of pages in this book is permitted only under license from ACS. Direct these and other permission requests to ACS Copyright Office, Publications Division, 1155 16th Street, N.W., Washington, DC 20036.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.fw001

Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

ACS Books Department

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.pr001

Preface

The human tendency to regard little things as important has produced very many great things.
- Georg C. Lichtenberg

The use of antimicrobial peptides (AMPs) as agents in therapeutic and agricultural applications is nearing reality. From the earliest characterization of their antimicrobial activities to novel methods for their synthesis and high-throughput screening assays, this class of antimicrobial agents has been the driving force behind the development of next-generation therapeutics and preventative medicines as well as in the control of plant diseases. More than 1700 natural AMPs from a wide range of life forms ranging from prokaryotes to humans have been characterized to date and many of these have served as templates for the rational design of synthetic peptides with improved potency, specificity, stability, and bioavailability. Once thought to be very uniform in their antimicrobial activity as membrane disruptants, research is now revealing the multifunctional nature of these molecules as critical players not only in the host's innate immunity but also as modulators of the adaptive immune response in mammals or as elicitors of defense responses in plants. Several AMPs not only function solely through interaction and disruption of pathogen cellular membranes but also have the ability to cross the pathogen's cell membrane in a nondestructive manner and interact with intracellular targets interfering with important biological functions and leading to cell death.

AMP technology has tremendous implications for the development of novel therapeutics and plant protective strategies. This book assembles contributions by internationally acclaimed scientists with a focus on therapeutic and agricultural applications. Promising medical applications of peptide technology include treatments for bacterial, fungal and viral infections. Production of more effective peptides, targeting the treatment of cancer, utilizes novel strategies for designing peptide immunogens to elicit specific antibodies. In humans, peptides that modulate the host's adaptive immune response will not be recognized by the invading pathogen as a defense factor and therefore will not be prone to development of resistance by the pathogen. Agricultural applications include control of devastating plant diseases caused by microbial pathogens, some of them resulting in mycotoxin contamination of food and feed products. Though plants and their various pathogens wage a continuous war of resistance against one another, transgenic strategies that utilize AMPs with different modes of action (both extra- and intracelluarly) against target pathogens should provide a

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

self defense mechanism that will be much harder for the pathogen to circumvent and develop resistance.

This book is the result of the symposium "Small Wonders: Peptides for Disease Control" held at the 240th National Meeting of the American Chemical Society in Boston, MA, August 22-26, 2010. To our knowledge, this is the first book that covers a broad range of peptide technology and its practical application in the agricultural, medical, and pharmaceutical fields. The up-to-date reviews and original research presented in this book will be of interest to a diverse audience including scientists in the medical, pharmaceutical, agricultural, chemical and biotechnological fields. Biochemists, molecular biologists, microbiologists and graduate students engaged in research and the development of peptides will find this book a useful reference tool. In summary, this book will represent a timely and much-needed comprehensive update of the literature in the field of peptides for disease control, an important and fast-moving subject area.

We are thankful to the authors for their enthusiastic participation in the symposium and timely contribution to this volume. We would be remiss if we did not acknowledge the immense help of several reviewers who helped us in expediting the thorough peer review process. Thanks are also due to the ACS Division of Agriculture and Food Chemistry (ACS-AGFD) for sponsoring the symposium and for the generous financial assistance from ACS-AGFD and AgroMed LLC. We also appreciate the following members of the ACS Publication Division for the editorial assistance - Tim Marney, Bob Hauserman, Arlene Furman, and Mary Calvert and for the cover design by Pamela Kame.

Kanniah Rajasekaran
Research Biologist
Food and Feed Safety Research Unit
Southern Regional Research Center
USDA Agricultural Research Service
1100 Robert E. Lee Blvd.
New Orleans, LA 70124, U.S.A.
+1 (504) 286-4482 (telephone)
+1 (504) 286-4533 (fax)
Rajah.Rajasekaran@ars.usda.gov (e-mail)

Jeffrey W. Cary
Molecular Biologist
Food and Feed Safety Research Unit
Southern Regional Research Center
USDA Agricultural Research Service
1100 Robert E. Lee Blvd.
New Orleans, LA 70124, U.S.A.
+1 (504) 286-5264 (telephone).
Jeff.Cary@ars.usda.gov (e-mail)

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.pr001

Jesse M. Jaynes
Professor of Biochemistry
Tuskegee University
Tuskegee, AL 36088, U.S.A.
+1 (678) 787-2416 (telephone)
jjaynes@tuskegee.edu (e-mail)

Emilio Montesinos
Professor of Crop Science (Plant Pathology)
Institute of Food and Agricultural Technology
University of Girona
Campus Montilivi, s/n
17071 Girona, Spain
+34 972 418427 (telephone)
emilio.montesinos@udg.edu (e-mail)

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.pr001

Downloaded by CORNELL UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ot001

Editors' Biographies

Kanniah Rajasekaran

Kanniah Rajasekaran is a Research Biologist with the Food and Feed Safety Research Unit, Agricultural Research Service, USDA in New Orleans, LA. He is an international authority on application of aseptic cell/tissue culture and recombinant DNA technologies towards genetic improvement of food and feed crops. He received his B.S. and M.S. in Agricultural Sciences from Tamil Nadu Agricultural University, Coimbatore, India and Ph.D. in Crop Biotechnology from the University of Sydney, Australia. He moved to his current position with the USDA, ARS in 1996 after a considerable industry experience with Phytogen, a plant biotechnology company in Pasadena, California. Current research interests include host-pathogen relationship, antifungal proteins and peptides, development of transgenic cotton/corn/peanut varieties with resistance to phytopathogens including mycotoxin-producing fungi, and tissue- or organelle-specific expression of transgenes.

Jeffrey W. Cary

Jeffrey W. Cary is a Molecular Biologist in the Food and Feed Safety Research Unit in New Orleans, which has received international recognition for its work in the elucidation of the genes involved in aflatoxin biosynthesis and their regulation. He earned his Ph.D. in Microbiology from Louisiana State University in 1986 and subsequently performed postdoctoral research on the molecular biology of the *Clostridium acetobutylicum* acetone/butanol fermentation at Rice University. He joined the USDA, ARS, Southern Regional Research Center at New Orleans in 1989 where he has been studying the regulation of fungal development and toxin production as well as engineering plants for expression of novel antimicrobial peptides/proteins as a means to control aflatoxin contamination in food and feed crops. Dr. Cary has appeared as an author in 110 publications and served as Chair of (2003−04) of the Fermentation and Biotechnology Division of the American Society for Microbiology.

Jesse M. Jaynes

Jesse M. Jaynes is a Professor of Biochemistry at Tuskegee University where he does research and teaches biochemistry in the Integrative Biosciences Program. He received his associate degree in anthropology from College of Eastern Utah, B.S. degree in biology from Southern Utah State College and Ph.D. in chemistry from Brigham Young University mentored by Leo Vernon. He received an NSF

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

post-doctoral fellowship to work with Ray Valentine at University of California, Davis. Another post-doctoral fellowship was spent at Montana State University. He spent the next 10 years at Louisiana State University and then moved on and founded a biotechnology company to design novel synthetic peptides.

Emilio Montesinos

Emilio Montesinos is a Professor of Microbiology since 1985 (Autonomous University of Barcelona, Spain) and of Plant Pathology since 1993 (University of Girona, Spain). He was also a Visiting Professor at the Department of Plant Pathology of Cornell University in 1994. His research focus is on Plant Microbiology and Plant Pathology, specifically in the area of novel control methods of plant diseases using microbial biopesticides and synthetic antimicrobial peptides. He has been invited to present in several conferences including International Society for Plant Pathology and American Chemical Society. He is an Honorary Member of the Spanish Society for Plant Pathology and served as its President (2004−2007).

Downloaded by CORNELL UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ot001

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Chapter 1

Immunomodulatory Cationic Peptide Therapeutics: A New Paradigm in Infection and Immunity

**Neeloffer Mookherjee,[1] Leola N. Y. Chow,[1]
and Robert E. W. Hancock*,[2]**

**[1]Manitoba Centre for Proteomics and Systems Biology, Department of Internal Medicine, University of Manitoba, Winnipeg, MB
[2]Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, BC, Canada
*E-mail: bob@cmdr.ubc.ca**

Natural cationic host defence (antimicrobial) peptides are widely distributed gene encoded molecules with diverse structures. There are more than 1200 natural Host Defence Peptides (HDPs) described to date. Due to the multifunctional roles defined for such peptides there is a keen interest in the potential therapeutic applications of HDPs and their synthetic mimics, Antimicrobial peptides and Innate Defence Regulator (IDR) peptides. These peptides constitute two broad classes of potential therapeutics; (i) with direct antimicrobial and/or anti-biofilm activity, and (ii) with immune-modulating and/or anti-inflammatory activity. Exploiting the immunomodulatory functions of these peptides represents a new therapeutic approach for resolution of infections and inflammatory disorders.

Introduction

More than two decades ago cationic peptides, discovered in the skin of frogs, lymph of insects and in human neutrophils, were demonstrated to be actively antimicrobial compounds (*1*). Even though cationic host defence (antimicrobial) peptides were initially defined as natural microbicidal agents, it is now increasingly appreciated that collectively these peptides are multifunctional immune effector

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

and regulatory molecules that protect against infections, maintain homeostasis, support healing while suppressing potentially harmful inflammation, and provide a functional link between innate and adaptive immunity (*2*). Therefore here we use the collective term Host Defence Peptides (HDPs), which accurately encompasses their diverse biological functionality while the more common term Antimicrobial peptides (AMPs) is used only to describe direct antibiotic activity.

HDPs are gene encoded ribosomally synthesized molecules, typically 12-50 amino acids in length with a net positive charge ranging from +2 to +7 with ≥ 30 % hydrophobic residues (*3*). Based on their conformational structures in membrane-like environments, these peptides can be broadly divided into four categories; amphipathic α-helix (e.g. cathelicidin CRAMP), β-sheets stabilized by disulphide bridges (e.g. protegrin), peptides with extended structures (e.g. indolicidin), and peptides with loop structures (e.g. bactenecin) (*4*) (Fig. 1). HDPs are widely distributed in Nature, being found in plants, insects and mammals. Well characterized families of HDPs in vertebrates are the cathelicidins and defensins defined by their conserved prepro sequences and semi-conserved disulphide arrays respectively. HDPs are expressed in cell types such as phagocytic leukocytes, epithelial cells and keratinocytes, and in a most tissues and body fluids (*5–8*). There are more than 1200 natural HDPs described to date, with >900 defined from eukaryotes (http://aps.unmc.edu/AP/main.php). The vast repertoire of natural HDPs thus provides an extensive template for the design of short synthetic derivatives. These synthetic derivatives can be designed to maintain or enhance biological activity with limited associated host cytotoxicity and are known as Antimicrobial (AMPs) or Innate Defence Regulator (IDR) peptide (*9–11*). Traditional development approaches have concentrated on developing directly antibiotic, topically-applied AMPs. However there is an increasing appreciation that the IDR peptides show much more promise for systemic usage. As these peptides protect against a wide range of infections, and confer anti-infective immunity by modulating innate and adaptive immune responses, there is a growing interest in the therapeutic development.

AMPs are well described elsewhere (*1–4*, *12*). Basically there have been a broad range of clinical trials on these molecules that effectively mimic two well established bacterial-derived cationic peptide drugs polymyxin B and gramicidin S. However although one, Omiganan, showed statistically significant activity in Phase III clinical trials as a topical agent to prevent catheter colonization and tunnel infections, none have as yet been awarded new drug approval. Newer methods of peptides screening and production are leading to broad spectrum antimicrobial peptides with excellent *in vitro* activity that are short and/or protease resistant (*1*). The basis for protection may be more complex than previously thought since Omiganan has also demonstrated significant efficacy in Phase II trials against Rosacea, an inflammatory non-infectious skin condition. This appears to indicate that even AMPs have the potential to work as immune modulators. Other avenues for exploitation of the action of cationic peptides on bacteria include the ability of some peptides to reduce biofilm formation at sub-MIC concentrations (*13*) and their ability to retain antimicrobial activity even when covalently bound to surfaces (*14*).

There are at least three avenues where the potential of immunomodulatory HDPs can be exploited for therapeutic development. It has been demonstrated that HDPs and IDR peptides with no direct microbicidal activity can protect against a wide variety of infections, through selective modulation of the innate immune response (*9*, *15*, *16*). This provides a distinct advantage in developing these molecules as therapeutics to treat infections that can circumvent problems of antimicrobial resistance. Non-microbicidal cationic peptides that protect against infections through their immunomodulatory properties do not exert selective pressure to develop resistance as they are directed at the host rather than the pathogen and work by selectively enhancing host immune mechanisms. It is extremely likely that such a treatment would be developed to treat infections as an *adjunctive* therapy in combination with conventional antibiotics (*16–18*). Secondly, the ability of HDPs and IDR peptides to suppress certain pro-inflammatory pathways and up-regulate anti-inflammatory mechanisms while maintaining efficient innate immune responses (*9*, *16*, *19*), makes them useful as potential anti-inflammatories for acute and chronic inflammatory disorders, and to suppress pathogen-induced inflammation. These could serve as therapeutics agents that might limit the escalation of inflammation without compromising host immunity. Third, HDPs and IDR peptides, through their action on innate immunity, have been demonstrated to modulate the adaptive immune response (*20–22*) and thus can be developed as potential adjuvants for vaccines (*11*, *23*, *24*). Table I summarizes some of the cationic peptide-based therapeutics in clinical development. In this chapter we discuss design strategies for IDR peptides, and summarize the progress and challenges associated with the development of HDPs and IDR peptides as anti-infective and immunomodulatory therapeutics and adjuvants.

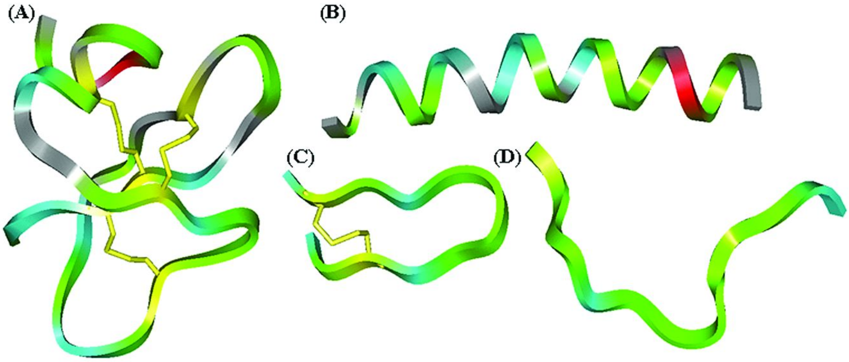


Figure 1. ***Structures of cationic peptides.*** *Cationic peptides can be broadly divided into four categories;* ***(A)*** *peptides with loop structures,* ***(B)*** *amphipathic α-helix,* ***(C)*** *β-sheets stabilized by disulphide bridges and* ***(D)*** *peptides with extended structures.*

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Table I. Host defence peptide-based therapeutics in clinical development (*84*)

Peptide-Based Drug	***Company***	***Trial Phase***	***Proposed Clinical Use***
Omiganan (MX-226 / MBI-226)	Migenix	III & II	Treatment of catheter infections, topical antiseptic, and anti-inflammatory for acne and rosacea.
Pexiganan acetate (MSI-78)	MacroChem	III	As topical antibiotic.
Iseganan (IB-367)	Ardea Biosciences	III	Treatment of oral mucositis in radiation therapy patients.
Delmitide (RDP58)	Genzyme	Post II	Treatment of inflammatory bowel disease.
hLF1-11	AM Pharma	I / II	Treatment of fungal infections and bacteremia in immunocompromised patients e.g. patients undergoing hematopoetic stem cell transplants.
Opebacan	Xoma	I / II	For endotoxemia in recipients of hematopoetic stem cell transplants.
PAC-113	Pacgen Biopharmaceuticals	II	Treatment of fungal infections.
AP-214	Action Pharma A/S	II	Treatment of sepsis and use in post-surgical organ failure.
CD-NP	Nile Therapeutics	II	For use in organ failure.
Ghrelin	Miyazaki University, Japan Papworth Hospital, UK.	II	Treatment of airway inflammation, chronic respiratory infections and in cystic fibrosis.
OP-145	OctoPlus N.V.	II	Treatment of chronic bacterial otitis media.
Xoma-629	Xoma	IIa	Impetigo.
CZEN-002	Zengen	IIb	Treatment of vulvovaginal candidiasis.
Hexapeptide-7	Helix BioMedix	I	For wound healing and skin regeneration.
Vasoactive intestinal peptide (VIP)	State University of New York	I	Treatment of respiratory tract infections and of sepsis.

Continued on next page.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Table I. (Continued). Host defence peptide-based therapeutics in clinical development (*84*)

Peptide-Based Drug	*Company*	*Trial Phase*	*Proposed Clinical Use*
IMX942	Inimex	Ia	Treatment of nosocomial infections and in febrile neutropenia.
PMX-30063	PolyMedix	Ib	As an antibiotic.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Synthetic Variants: Antimicrobial and Innate Defence Regulator (IDR) Peptides

Traditional approaches to peptide design have involved systematic variations in the structure of a base molecule, usually to optimize a limited range of parameters such as cationic charge, hydrophobicity, and hydrophobic moment. When performed in conjunction with structural modelling or structure determination of the base molecule, such design methods can yield useful increases in activity (*1*). Although this approach was used for almost all clinically developed peptides to date, there are some limitations for this approach including (i) each amino acid change in a small peptide yields a change in secondary structure making it nearly impossible to accurately relate activity to structure, this is especially concerning since the same pair of adjacent amino acids will have very different atomic properties when sited within different secondary structures (e.g. α-helices, β-sheets or turns, polyproline helices and random structures, all of which have been found in natural HDPs) (*25*), (ii) the starting structure effectively guides the final output, and to some extent limits the value of this approach, as it limits molecular diversity, (iii) such optimizations are usually limited to tens of peptides whereas up to 10,000 compounds are required to enable development of successful drugs, and (iv) there are many more structural parameters that are influential than the three properties discussed above (*26*). A game changer was the development of technologies for much higher throughput, cost effective production of small peptides using robotic synthesis on peptide arrays (so called SPOT synthesis) (*27*). This enables broad screening and the rapid development of optimized peptides when used in combination with newer approaches involving chemi-informatics. In these procedures, the structural properties of peptides (determined by a series of conventional and inductive “descriptors” that are calculated from the primary sequence and are sensitive to structure) were related, using machine learning approaches, to measured activities, and used to quite accurately predict the activity of a 100,000 virtual peptides. Application of these procedures led to the identification of 9 amino acid peptides with broad spectrum activity against many pathogens, superior activity against highly resistant Superbugs than conventional agents, and an ability to protect against systemic infections (*26*).

Similar procedures have not been pursued with IDR peptides and traditional and random design approaches predominate. In this case, we have found that chemokine induction by monocytes is a reasonable surrogate for anti-infective immunomodulatory activity (*28*), whilst suppression of LPS-induced TNF-α production works to screen for anti-inflammatory properties (*19*).

Cationic Peptides as Broad Spectrum Antimicrobials

Many HDPs including cathelicidins, defensins and hepcidin, have been demonstrated to protect against bacterial, viral and parasitic infections (*15*, *29–34*). Several studies provide evidence to correlate the expression of HDPs with susceptibility or resistance to bacterial infections (*35–37*). Although these studies are often interpreted as being due to direct antimicrobial activity, the data often does not discriminate between this and stimulation of protective innate immunity. Lack or low expression of certain HDPs in humans results in increased susceptibility to infections. For example, patients with morbus Kostmann have deficiencies in cathelicidin-LL37 and α-defensins HNP1-3 and suffer from frequent periodontal infections (*36*). Similarly, patients with specific granule deficiency display an almost complete deficiency of defensins, and suffer from frequent severe bacterial infections (*38*). In contrast, in animal studies, mice expressing human LL-37 or human defensin 5 (HD-5) show increased resistance to bacterial challenge (*37*, *39*). Similarly, the lantibiotic duramycin has been demonstrated to be effective as a potential treatment in cystic fibrosis (*40*). It has also been suggested that vitamin D-mediated induction of human HDP LL-37 contributes to innate immune responses to infections and wounds, in that the CAMP gene which encodes for human cathelicidin LL-37 was shown to be a direct target of vitamin D / vitamin D receptor complex and increased susceptibility to infections associated with vitamin D deficiency may thus be due to the lack of appropriate HDP expression (*41–43*). Taken together it is apparent that the absence of one or more HDPs leads to increased susceptibility to infections, while induction or exogenous introduction of HDP protects against infections.

In general, when HDPs are present at very high concentrations, such as in the granules of phagocytes, in intestinal crypts or adjacent to degranulating phagocytes, they might have direct antimicrobial properties (*15*, *16*, *21*); however most HDPs are strongly antagonized by physiological divalent cation concentrations (2 mM Mg^{2+}, Ca^{2+}) and anionic polysaccharides like heparin (*15*). Mechanistically, polycationic AMPs work against Gram negative bacteria by binding to the polyanionic lipopolysaccharide (LPS) on the surface bilayer of the bacterial outer membrane, followed by translocation by the self promoted uptake mechanism (*44–47*). Then they bind to the outer monolayer of the cytoplasmic membrane and at appropriate concentrations trigger localized perturbations of the membrane, as described in a variety of different models (*47*). The actual lethal event differs between peptides and target organisms and seems to involve considerable complexity, involving often several of the following: disruption of membrane integrity, collapse of membrane potential and loss of intracellular

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

pH homeostasis, interference with membrane associated biosynthetic enzymes involved in e.g. cell wall biosynthesis and cell division, and/or translocation into the cell and inhibition of cytoplasmic functions including macromolecular synthesis and the function of specific enzymes (*47*, *48*). These events all likely involve relatively low affinity interactions with targets that complement the cationic amphipathic HDPs in being anionic or hydrophobic, explaining the ionic inhibition of HDP activity. Therefore, it has been proposed that for those HDPs that are strongly antagonized by physiological salt concentrations or are present in relatively low levels, their anti-infective protective functions might be largely due to the modulation of immune responses in the host (*15*, *17*, *19*, *29*, *49*), since immunomodulatory functions occur readily at physiological salt concentrations (such as those found in tissue culture medium and *in vivo*). It has also been demonstrated that a synthetic IDR-1 derivative of bovine bactenecin, without any direct antimicrobial activity, confers protection in several animal models of bacterial infection (*9*). Similarly in a mouse model of *Pseudomonas aeruginosa* infection, a truncated version of human cathelicidin peptide LL-37 was able to decrease the level of bacterium-induced injury (*50*). Other immunomodulatory IDR peptides, in particular IDR-1002, have been demonstrated to be protective against a range of infections in animal models (*10*). Consistent with this, a wide range of immunomodulatory functions have been demonstrated to be mediated by natural HDPs and IDR peptides both *in vitro* and in animal models, including direct and indirect recruitment of critical immune cells, modulation of cytokine and chemokine production, anti-endotoxin and anti-inflammatory activities, barrier repair and wound healing, and modulation of dendritic cell differentiation and T-cell polarization (*9*, *10*, *12*, *19*, *22*, *51–53*). Mechanistic studies have demonstrated that such interactions are complex with a number of receptors, intracellular uptake, and several pathways and transcription factors controlling the expression of hundreds of genes.

The immunomodulatory functions of HDPs contributing to anti-infective immunity cannot be considered in isolation as HDPs have been shown to work in synergy with other immune effector molecules. For example, HDP such as human LL-37 can function synergistically with cytokines including the granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-1β (*54*, *55*). The presence of GM-CSF increases the magnitude of LL-37-induced phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2) and p38 MAPK in peripheral blood-derived monocytes and thus may reduce the threshold concentration of LL-37 required to activate these pathways (*15*, *54*). MAPK ERK1/2 and p38 are involved in various immune responses including initiation of innate immunity and activation of adaptive immunity (*56*). Therefore, it is likely that during an infection, HDPs can act synergistically with specific cytokines to amplify immunomodulatory effects required for the overall resolution of infections.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Cationic Peptides as Selective Immunomodulatory Agents

The biological roles of HDPs include a wide range of immunomodulatory functions (*12*, *51*). It is thus not surprising that dysregulation or altered HDP expression has been linked to various immune-mediated chronic inflammatory diseases. For example decreased expression of human β-defensins is associated with the pathogenesis of inflammatory bowel disease, Crohn's in children and psoriatic plaques (*57–59*). Similarly, reduced expression of cathelicidin LL-37 and dermcidin are linked to increased risk of atopic dermatitis (*60*, *61*). In contrast, over expression of LL-37 is linked to psoriasis (*62*), and increased accumulation of defensins is seen in the synovial fluid of patients with rheumatoid arthritis (*63*). Simiarly several studies using transgenic mouse models and bioengineered tissues have demonstrated that cationic peptides not only can protect against various infections but also contribute significantly to resolution of inflammation (reviewed in Dybvig et al, 2011 (*64*)). Consistent with this several studies have shown that HDP and IDR peptides can 'selectively' regulate inflammatory processes, enhancing certain pro-inflammatory pathways such as chemokine expression, immune cell recruitment, cellular differentiation and other responses required for the resolution of infections, while suppressing pro-inflammatory cytokine production in response to bacterial TLR agonists and up-regulating anti-inflammatory mechanisms (*9*, *10*, *16*, *19*, *23*, *49*, *53*, *65–67*) (Fig. 2).

Previous studies have demonstrated surface binding, cellular uptake and endocytic mobilization of HDP in monocytic cells and epithelial cells, and has suggested that cellular uptake is essential for the immunomodulatory activities such as chemokine induction (*68*, *69*). Both intracellular interacting protein partners, like SQSTM-1 and GAPDH, and cell surface receptors, including various Gi-coupled receptors, have also been described for HDP such as cathelicidin LL-37 and IDR peptides (*10*, *68*, *70*, *71*). However, the mechanisms of receptor interaction for HDP and IDR peptides are yet to be completely resolved. It is possible that there are a variety of moderate affinity receptors rather than a single high affinity receptor. After binding to the membrane or surface receptors, an atypical endocytic uptake pathway appears to facilitate the internalization of HDP and IDR peptides, in a manner analogous to the structurally related cell penetrating peptides (*69*, *72*, *73*), followed by interaction with the intracellular receptors (*68*, *70*). These interactions appear to facilitate modulation of immune signalling pathways, both in the absence and presence of a subset of endogenous immune effectors or exogenous bacterial TLR agonists, resulting in the 'selective' modulation of inflammatory responses.

Endotoxin-induced specific inflammatory responses such as TNF-α, IL-1β and IL-6 production, NF-κB1 (p105/p50) and TNF-α-induced protein-2 (TNFAIP2) expression, and the activation of NF-κB/Rel family of transcription factors, which plays a critical role in the inflammatory process are significantly suppressed by HDPs and IDR peptides (*9*, *19*, *66*). HDPs can also influence key signalling pathways such as MAPK ERK1/2, PI3 kinase, and AP-1 etc (*66*). In contrast, HDP were shown to maintain or enhance cellular responses that antagonize inflammation such as the expression of TNF-α-induced protein-3 (TNFAIP3/A20) and anti-inflammatory mediators such as IL-10, and the

NF-κB inhibitor NFκBIA (*9*, *10*, *19*, *66*). HDP can induce the production of chemokines, for example MCP-1, IL-8 and several others, and up-regulate the surface expression of chemokine receptors such as for example IL-8RB and CXCR-4, in various cell types suggesting that these peptides promote immune cells recruitment (*52*). Indeed, HDP can either directly or indirectly promote recruitment of a variety of immune cells including neutrophils, monocytes, immature dendritic cells, mast cells, T-cells, eosinophils and neutrophils (*16*, *21*, *23*, *71*). In addition, HDPs can directly influence cellular differentiation and modification. For example, human cathelicidin LL-37 was shown to up-regulate the endocytic capacity of premature dendritic cells and modify the expression of phagocytic receptors and enhance the secretion of Th-1 inducing cytokines in mature dendritic cells (*22*). It has also been suggested that HDPs, in particular cathelicidin peptides, can influence brain immunity by stimulating glial cell activation, cytokine production and aid brain cell protection by inducing neurotrophic factors (*74*). Other immunomodulatory roles associated with HDPs include mast cell stimulation (*75*), promotion of angiogenesis (*76*) and wound healing (*77*).

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

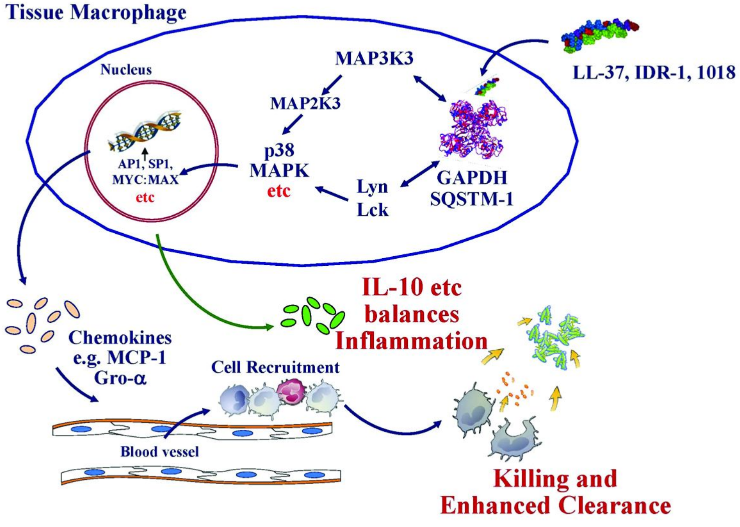

Tissue Macrophage
Nucleus
MAP3K3
MAP2K3
p38
MAPK
etc
LL-37, IDR-1, 1018
AP1, SP1,
MYC:MAX
etc
Lyn
Lck
GAPDH
SQSTM-1
IL-10 etc
balances
Inflammation
Chemokines
e.g. MCP-1
Gro-α
Cell Recruitment
Blood vessel
Killing and
Enhanced Clearance

Figure 2. ***Mechanism of action of immunomodulatory HDPs and IDR-peptides.*** *Internalization of HDPs and IDR peptides is facilitated by an atypical endocytic uptake, followed by interaction with the intracellular receptors. These interactions appear to facilitate modulation of various immune signalling pathways, mediates various immunomodulatory responses and overall results in the 'selective' modulation of inflammatory responses. Modified from J. Immunol. 183, 2688-2696 (2009), Mol. Biosystems 5, 483-496 (2009) and J. Biol. Chem. 284, 36007-36011 (2009).*

Overall, the diverse and paradoxical immunomodulatory functions exhibited by HDP can lead to rebalanced / controlled inflammation with a net anti-infective response in the host. This suggests that HDPs and IDR peptides might also be promising therapeutic agents to treat immune-mediated inflammatory disorders. An important consideration regarding current therapeutics used for chronic inflammatory diseases is the increased associated risk of infections and neoplasms due to compromised immune functioning (*78*, *79*). The targeted anti-inflammatory function of HDPs and IDR peptides makes them attractive candidates as potential therapeutics for chronic inflammatory disorders. A distinct advantage of developing these peptides as anti-inflammatory agents is their potential to selectively suppress escalation of inflammation without hampering innate immune responses required for resolution of infections.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Cationic Peptides as Vaccine Adjuvants

The ability of HDPs to modulate aspects of the innate immune system has made them potential candidates as vaccine adjuvants, since it is well known that innate immunity instructs adaptive immunity. Thus the appropriate stimulation of innate immunity promotes a transition to enhanced and appropriately polarized antibody or cellular immune responses to foreign antigens. The HDP activities mentioned above involving the regulation of cytokine responses, enhancing and modulating DC and lymphocyte recruitment and maturation, as well as T_H cell polarization, all play a major role in the development of an effective adaptive immune response. Animal studies have shown that the use of human neutrophil defensins and LL-37 as adjuvants led to significant enhancement of adaptive, antigen-specific, immunity (*80*, *81*). Recent studies have investigated the effects on adaptive responses by IDR peptides used in combination with CpG ODNs. Indolicidin, a bovine HDP, and its analogs when co-formulated with CpG ODN and polyphosphazene, significantly enhanced antigen-specific humoral responses and promoted cell-mediated immunity in cattle, compared to CpG ODN with emulsigen®, an adjuvant that is often used in veterinary vaccines (*82*). In this instance it was suggested that the polyphosphazene created a depot, peptides enhanced immune cell recruitment, and CpG led to activation of those immune cells. Similarly, IDR-HH2 peptide in complex with CpG ODN, within a pertussis toxoid vaccine formulation, synergistically induced the production of chemokines and significantly enhanced the production of protective toxoid-specific antibodies in mice (*83*). This formulation demonstrated responses indicative of a balanced T_H1/T_H2 response. Intriguingly, potent immune responses were observed even after a single application of adjuvanted pertussis toxoid and animals became protected against pertussis infections with this formulated vaccine. These studies demonstrate the strong potential for using HDPs and IDR peptides as vaccine adjuvants to promote an effective, long-lasting and balanced protective response.

Emerging Technologies Facilitating the Development of Cationic Peptide Therapeutics

AMPs have already navigated their way through clinical trials and although they have shown efficacy in Phase III trials, none has to date obtained new drug approval. IDR peptides are also in clinical trials Phase I/II (*84*). Some challenges in the development of AMP and IDR peptide therapeutics are bioavailability, potential toxicity, usage systemically, and manufacturing costs. These areas that need to be addressed for the development of cationic peptides as viable therapeutics. Some HDPs may be liable to proteases (*12*), for example chymotrypsin-like enzymes can attack proteins at basic residues that are a hallmark feature of HDPs (*12*). IDR peptides appear to be effective even in the face of this concern. Several solutions to resolve this issue has been proposed. For example, the use of unusual or D-(rather than natural L-) amino acids, the development of cyclic peptides with strained peptide bonds, or chemical modification of peptides to create protease resistant molecules can be employed (*1*, *12*, *85*, *86*). Alternatively, improved formulations such as in liposomes to mask the peptide and the use of non-peptidic backbones to create protease-resistant mimetics could also help to resolve sensitivity to proteases (*12*, *85*, *86*). These approaches could also assist in making peptides work systemically. Also, it has been documented that high concentrations of certain HDPs are cytotoxic to a variety of eukaryotic cell types (*21*). For example, HNP-1 induces progressive lung dysfunction in a dose dependent manner in mice (*87*). Nevertheless it seems possible to make peptides with low toxicity in animal models, although there is a lack of published toxicology data in animals. Finally, The high cost of manufacturing HDPs is a significant challenge, as the laboratory and commercial scale costs of even modest sized peptides can range from $100 to $600 per gram which is an average daily dose for most systemic applications (*12*, *88*). Nevertheless even these issues are likely to be overcome as the development of effective small peptides of 9-12 amino acids (*9*, *10*, *26*), reductions in commercial scale costs, and new recombinant methods (*89*), all have the potential for substantially lowering costs. Thus the focus in the development of HDPs for clinical applications is on small peptides, performing extensive structure activity relationship studies to assist in limiting potential toxicity, and lowering the cost of drug production.

Summary/Conclusion

HDPs and synthetic derivative AMPs and IDR peptides are rapidly emerging as potential novel therapeutics that can directly kill pathogens and/or modify immune responses to control infections and inflammation. Apart from their anti-infective properties, a wide range of immunomodulatory functions have been defined for HDPs and IDR peptides that result in a net suppression of potentially harmful pro-inflammatory responses along with enhancement of effective immunity enabling resolution of infections. The multiple molecular modes of action associated with these peptides make these attractive candidates as potential therapeutics for at least four clinical avenues; as direct antimicrobials and anti-biofilm agents, as anti-inflammatories, in wound healing and as adjuvants.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

The distinct advantages of developing these cationic peptides as therapeutics are two fold; (i) their ability to circumvent or avoid problems of microbial resistance, and (ii) their frequent ability to control inflammation without compromising the host's anti-infective immunity. However, there are some challenges in the process of developing peptide therapeutics, essentially limited bioavailability, unknown toxicities and high cost of production. Future directions in the development of cationic peptide therapeutics would perhaps focus on short IDR peptide derivatives of HDPs, with optimization of desired biological activities and limited cytotoxicity, while exploring the best mode of delivery to make the peptides bioavailable. Overall cationic AMPs and IDR peptides represent an exciting new approach as immunomodulatory therapeutics.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Acknowledgments

REWH is funded for his peptide research by Canadian Institutes of Health Research (CIHR) and he holds a Canada Research Chair. NM is funded by Manitoba Health Research Council (MHRC) and Health Sciences Centre Foundation, Manitoba, Canada, for peptide research.

References

1. Hadley, E. B.; Hancock, R. E. W. Strategies for the discovery and advancement of novel cationic antimicrobial peptides. *Curr. Top. Med. Chem.* **2010**, *10* (18), 1872–81.
2. Bevins, C. L.; Salzman, N. H. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat. Rev. Microbiol.* **2011**, *9* (5), 356–68.
3. Hancock, R. E. W.; Chapple, D. S. Peptide antibiotics. *Antimicrob. Agents Chemother.* **1999**, *43* (6), 1317–23.
4. Lai, Y.; Gallo, R. L. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol.* **2009**, *30* (3), 131–41.
5. Murakami, M.; Ohtake, T.; Dorschner, R. A.; Gallo, R. L. Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. *J. Dent. Res.* **2002**, *81* (12), 845–50.
6. Murakami, M.; Ohtake, T.; Dorschner, R. A.; Schittek, B.; Garbe, C.; Gallo, R. L. Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *J. Invest. Dermatol.* **2002**, *119* (5), 1090–5.
7. Murakami, M.; Dorschner, R. A.; Stern, L. J.; Lin, K. H.; Gallo, R. L. Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk. *Pediatr. Res.* **2005**, *57* (1), 10–5.
8. Phadke, S. M.; Deslouches, B.; Hileman, S. E.; Montelaro, R. C.; Wiesenfeld, H. C.; Mietzner, T. A. Antimicrobial peptides in mucosal secretions: the importance of local secretions in mitigating infection. *J. Nutr.* **2005**, *135* (5), 1289–93.

9. Scott, M. G.; Dullaghan, E.; Mookherjee, N.; Glavas, N.; Waldbrook, M.; Thompson, A.; Wang, A.; Lee, K.; Doria, S.; Hamill, P.; Yu, J. J.; Li, Y.; Donini, O.; Guarna, M. M.; Finlay, B. B.; North, J. R.; Hancock, R. E. W. An anti-infective peptide that selectively modulates the innate immune response. *Nat. Biotechnol.* **2007**, *25* (4), 465–72.
10. Nijnik, A.; Madera, L.; Ma, S.; Waldbrook, M.; Elliott, M. R.; Easton, D. M.; Mayer, M. L.; Mullaly, S. C.; Kindrachuk, J.; Jenssen, H.; Hancock, R. E. W. Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *J. Immunol.* **2010**, *184* (5), 2539–50.
11. Easton, D. M.; Nijnik, A.; Mayer, M. L.; Hancock, R. E. W. Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol.* **2009**, *27* (10), 582–90.
12. Hancock, R. E. W.; Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24* (12), 1551–7.
13. Overhage, J.; Campisano, A.; Bains, M.; Torfs, E. C.; Rehm, B. H.; Hancock, R. E. W. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect. Immun.* **2008**, *76* (9), 4176–82.
14. Hilpert, K.; Elliott, M.; Jenssen, H.; Kindrachuk, J.; Fjell, C. D.; Korner, J.; Winkler, D. F.; Weaver, L. L.; Henklein, P.; Ulrich, A. S.; Chiang, S. H.; Farmer, S. W.; Pante, N.; Volkmer, R.; Hancock, R. E. W. Screening and characterization of surface-tethered cationic peptides for antimicrobial activity. *Chem. Biol.* **2009**, *16* (1), 58–69.
15. Bowdish, D. M.; Davidson, D. J.; Lau, Y. E.; Lee, K.; Scott, M. G.; Hancock, R. E. W. Impact of LL-37 on anti-infective immunity. *J. Leukocyte Biol.* **2005**, *77* (4), 451–9.
16. Mookherjee, N.; Hancock, R. E. W. Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. *Cell. Mol. Life Sci.* **2007**, *64* (7-8), 922–33.
17. Hirsch, T.; Jacobsen, F.; Steinau, H. U.; Steinstraesser, L. Host defense peptides and the new line of defence against multiresistant infections. *Protein Pept. Lett.* **2008**, *15* (3), 238–43.
18. Steinstraesser, L.; Koehler, T.; Jacobsen, F.; Daigeler, A.; Goertz, O.; Langer, S.; Kesting, M.; Steinau, H.; Eriksson, E.; Hirsch, T. Host defense peptides in wound healing. *Mol. Med.* **2008**, *14* (7-8), 528–37.
19. Mookherjee, N.; Brown, K. L.; Bowdish, D. M.; Doria, S.; Falsafi, R.; Hokamp, K.; Roche, F. M.; Mu, R.; Doho, G. H.; Pistolic, J.; Powers, J. P.; Bryan, J.; Brinkman, F. S.; Hancock, R. E. W. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J. Immunol.* **2006**, *176* (4), 2455–64.
20. Oppenheim, J. J.; Biragyn, A.; Kwak, L. W.; Yang, D. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Ann. Rheum. Dis.* **2003**, *62* (Suppl 2), ii17–21.
21. Bowdish, D. M.; Davidson, D. J.; Hancock, R. E. W. A re-evaluation of the role of host defence peptides in mammalian immunity. *Curr. Protein Pept. Sci.* **2005**, *6* (1), 35–51.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

22. Davidson, D. J.; Currie, A. J.; Reid, G. S.; Bowdish, D. M.; MacDonald, K. L.; Ma, R. C.; Hancock, R. E. W.; Speert, D. P. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J. Immunol.* **2004**, *172* (2), 1146–56.
23. Bowdish, D. M.; Davidson, D. J.; Hancock, R. E. W. Immunomodulatory properties of defensins and cathelicidins. *Curr. Top Microbiol. Immunol.* **2006**, *306*, 27–66.
24. Cao, D.; Li, H.; Jiang, Z.; Xu, C.; Cheng, Q.; Yang, Z.; Cao, G.; Zhang, L. Synthetic innate defence regulator peptide enhances in vivo immunostimulatory effects of CpG-ODN in newborn piglets. *Vaccine* **2010**, *28* (37), 6006–13.
25. Powers, J. P.; Hancock, R. E. W. The relationship between peptide structure and antibacterial activity. *Peptides* **2003**, *24* (11), 1681–91.
26. Cherkasov, A.; Hilpert, K.; Jenssen, H.; Fjell, C. D.; Waldbrook, M.; Mullaly, S. C.; Volkmer, R.; Hancock, R. E. W. Use of artificial intelligence in the design of small peptide antibiotics effective against a broad spectrum of highly antibiotic-resistant superbugs. *ACS Chem. Biol.* **2009**, *4* (1), 65–74.
27. Hilpert, K.; Volkmer-Engert, R.; Walter, T.; Hancock, R. E. W. High-throughput generation of small antibacterial peptides with improved activity. *Nat. Biotechnol.* **2005**, *23* (8), 1008–12.
28. Wieczorek, M.; Jenssen, H.; Kindrachuk, J.; Scott, W. R.; Elliott, M.; Hilpert, K.; Cheng, J. T.; Hancock, R. E. W.; Straus, S. K. Structural studies of a peptide with immune modulating and direct antimicrobial activity. *Chem. Biol.* **2010**, *17* (9), 970–80.
29. Rajanbabu, V.; Chen, J. Y. Antiviral function of tilapia hepcidin 1-5 and its modulation of immune-related gene expressions against infectious pancreatic necrosis virus (IPNV) in Chinook salmon embryo (CHSE)-214 cells. *Fish Shellfish Immunol.* **2011**, *30* (1), 39–44.
30. Kindrachuk, J.; Scruten, E.; Attah-Poku, S.; Bell, K.; Potter, A.; Babiuk, L. A.; Griebel, P. J.; Napper, S. Stability, toxicity and biological activity of host defense peptide BMAP28 and its inversed and retro-inversed isomers. *Biopolymers* **2011**, *96* (1), 14–24.
31. Ohta, K.; Kajiya, M.; Zhu, T.; Nishi, H.; Mawardi, H.; Shin, J.; Elbadawi, L.; Kamata, N.; Komatsuzawa, H.; Kawai, T. Additive effects of orexin B and vasoactive intestinal polypeptide on LL-37-mediated antimicrobial activities. *J. Neuroimmunol.* **2011**, *233* (1-2), 37–45.
32. Arnett, E.; Lehrer, R. I.; Pratikhya, P.; Lu, W.; Seveau, S. Defensins enable macrophages to inhibit the intracellular proliferation of Listeria monocytogenes. *Cell Microbiol.* **2011**, *13* (4), 635–51.
33. Falco, A.; Ortega-Villaizan, M.; Chico, V.; Brocal, I.; Perez, L.; Coll, J. M.; Estepa, A. Antimicrobial peptides as model molecules for the development of novel antiviral agents in aquaculture. *Mini-Rev. Med. Chem.* **2009**, *9* (10), 1159–64.
34. Haines, L. R.; Hancock, R. E. W.; Pearson, T. W. Cationic antimicrobial peptide killing of African trypanosomes and Sodalis glossinidius, a bacterial

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

symbiont of the insect vector of sleeping sickness. *Vector Borne Zoonotic Dis.* **2003**, *3* (4), 175–86.

35. Morrison, G.; Kilanowski, F.; Davidson, D.; Dorin, J. Characterization of the mouse beta defensin 1, Defb1, mutant mouse model. *Infect. Immun.* **2002**, *70* (6), 3053–60.
36. Putsep, K.; Carlsson, G.; Boman, H. G.; Andersson, M. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet* **2002**, *360* (9340), 1144–9.
37. Salzman, N. H.; Ghosh, D.; Huttner, K. M.; Paterson, Y.; Bevins, C. L. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* **2003**, *422* (6931), 522–6.
38. Ganz, T.; Metcalf, J. A.; Gallin, J. I.; Boxer, L. A.; Lehrer, R. I. Microbicidal/cytotoxic proteins of neutrophils are deficient in two disorders: Chediak-Higashi syndrome and "specific" granule deficiency. *J. Clin. Invest.* **1988**, *82* (2), 552–6.
39. Bals, R.; Weiner, D. J.; Moscioni, A. D.; Meegalla, R. L.; Wilson, J. M. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect. Immun.* **1999**, *67* (11), 6084–9.
40. Jones, A. M.; Helm, J. M. Emerging treatments in cystic fibrosis. *Drugs* **2009**, *69* (14), 1903–10.
41. Liu, P. T.; Stenger, S.; Li, H.; Wenzel, L.; Tan, B. H.; Krutzik, S. R.; Ochoa, M. T.; Schauber, J.; Wu, K.; Meinken, C.; Kamen, D. L.; Wagner, M.; Bals, R.; Steinmeyer, A.; Zugel, U.; Gallo, R. L.; Eisenberg, D.; Hewison, M.; Hollis, B. W.; Adams, J. S.; Bloom, B. R.; Modlin, R. L. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **2006**, *311*, 5768, 1770–3.
42. Gombart, A. F. The vitamin D-antimicrobial peptide pathway and its role in protection against infection. *Future Microbiol.* **2009**, *4* (9), 1151–65.
43. Schauber, J.; Dorschner, R. A.; Coda, A. B.; Buchau, A. S.; Liu, P. T.; Kiken, D.; Helfrich, Y. R.; Kang, S.; Elalieh, H. Z.; Steinmeyer, A.; Zugel, U.; Bikle, D. D.; Modlin, R. L.; Gallo, R. L. Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J. Clin. Invest.* **2007**, *117* (3), 803–11.
44. Steinstraesser, L.; Kraneburg, U.; Jacobsen, F.; Al-Benna, S. Host defense peptides and their antimicrobial-immunomodulatory duality. *Immunobiology* **2011**, *216* (3), 322–33.
45. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415* (6870), 389–95.
46. Zhang, L.; Scott, M. G.; Yan, H.; Mayer, L. D.; Hancock, R. E. W. Interaction of polyphemusin I and structural analogs with bacterial membranes, lipopolysaccharide, and lipid monolayers. *Biochemistry* **2000**, *39* (47), 14504–14.
47. Hale, J. D.; Hancock, R. E. W. Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Rev. Anti-Infect Ther* **2007**, *5* (6), 951–9.

48. Bera, A.; Singh, S.; Nagaraj, R.; Vaidya, T. Induction of autophagic cell death in Leishmania donovani by antimicrobial peptides. *Mol. Biochem. Parasitol.* **2003**, *127* (1), 23–35.
49. Mookherjee, N.; Rehaume, L. M.; Hancock, R. E. W. Cathelicidins and functional analogues as antisepsis molecules. *Expert Opin. Ther. Targets* **2007**, *11* (8), 993–1004.
50. Sawa, T.; Kurahashi, K.; Ohara, M.; Gropper, M. A.; Doshi, V.; Larrick, J. W.; Wiener-Kronish, J. P. Evaluation of antimicrobial and lipopolysaccharide-neutralizing effects of a synthetic CAP18 fragment against Pseudomonas aeruginosa in a mouse model. *Antimicrob. Agents Chemother.* **1998**, *42* (12), 3269–75.
51. Gallo, R. L. Sounding the alarm: multiple functions of host defense peptides. *J. Invest. Dermatol.* **2008**, *128* (1), 5–6.
52. Scott, M. G.; Davidson, D. J.; Gold, M. R.; Bowdish, D.; Hancock, R. E. W. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J. Immunol.* **2002**, *169* (7), 3883–91.
53. Bowdish, D. M.; Davidson, D. J.; Scott, M. G.; Hancock, R. E. W. Immunomodulatory activities of small host defense peptides. *Antimicrob. Agents Chemother.* **2005**, *49* (5), 1727–32.
54. Bowdish, D. M.; Davidson, D. J.; Speert, D. P.; Hancock, R. E. W. The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. *J. Immunol.* **2004**, *172* (6), 3758–65.
55. Yu, J.; Mookherjee, N.; Wee, K.; Bowdish, D. M.; Pistolic, J.; Li, Y.; Rehaume, L.; Hancock, R. E. W. Host Defense Peptide LL-37, in Synergy with Inflammatory Mediator IL-1beta, Augments Immune Responses by Multiple Pathways. *J. Immunol.* **2007**, *179* (11), 7684–91.
56. Dong, C.; Davis, R. J.; Flavell, R. A. MAP kinases in the immune response. *Annu. Rev. Immunol.* **2002**, *20*, 55–72.
57. Wehkamp, J.; Schmid, M.; Stange, E. F. Defensins and other antimicrobial peptides in inflammatory bowel disease. *Curr. Opin. Gastroenterol.* **2007**, *23* (4), 370–8.
58. Zilbauer, M.; Jenke, A.; Wenzel, G.; Postberg, J.; Heusch, A.; Phillips, A. D.; Noble-Jamieson, G.; Torrente, F.; Salvestrini, C.; Heuschkel, R.; Wirth, S. Expression of human beta-defensins in children with chronic inflammatory bowel disease. *PLoS One* **2010**, *5* (10), e15389.
59. Peric, M.; Koglin, S.; Dombrowski, Y.; Gross, K.; Bradac, E.; Buchau, A.; Steinmeyer, A.; Zugel, U.; Ruzicka, T.; Schauber, J. Vitamin D analogs differentially control antimicrobial peptide/"alarmin" expression in psoriasis. *PLoS One* **2009**, *4* (7), e6340.
60. Ong, P. Y.; Ohtake, T.; Brandt, C.; Strickland, I.; Boguniewicz, M.; Ganz, T.; Gallo, R. L.; Leung, D. Y. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* **2002**, *347* (15), 1151–60.
61. Rieg, S.; Steffen, H.; Seeber, S.; Humeny, A.; Kalbacher, H.; Dietz, K.; Garbe, C.; Schittek, B. Deficiency of dermcidin-derived antimicrobial peptides in sweat of patients with atopic dermatitis correlates with an

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

impaired innate defense of human skin in vivo. *J. Immunol.* **2005**, *174* (12), 8003–10.

62. Gilliet, M.; Lande, R. Antimicrobial peptides and self-DNA in autoimmune skin inflammation. *Curr. Opin. Immunol.* **2008**, *20* (4), 401–7.
63. Bokarewa, M. I.; Jin, T.; Tarkowski, A. Intraarticular release and accumulation of defensins and bactericidal/permeability-increasing protein in patients with rheumatoid arthritis. *J. Rheumatol.* **2003**, *30* (8), 1719–24.
64. Dybvig, T.; Facci, M.; Gerdts, V.; Wilson, H. L. Biological roles of host defense peptides: lessons from transgenic animals and bioengineered tissues. *Cell Tissue Res.* **2011**, *343* (1), 213–25.
65. Scott, M. G.; Rosenberger, C. M.; Gold, M. R.; Finlay, B. B.; Hancock, R. E. W. An alpha-helical cationic antimicrobial peptide selectively modulates macrophage responses to lipopolysaccharide and directly alters macrophage gene expression. *J. Immunol.* **2000**, *165* (6), 3358–65.
66. Mookherjee, N.; Hamill, P.; Gardy, J.; Blimkie, D.; Falsafi, R.; Chikatamarla, A.; Arenillas, D. J.; Doria, S.; Kollmann, T. R.; Hancock, R. E. W. Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. *Mol. Biosyst.* **2009**, *5* (5), 483–96.
67. Mookherjee, N.; Wilson, H. L.; Doria, S.; Popowych, Y.; Falsafi, R.; Yu, J. J.; Li, Y.; Veatch, S.; Roche, F. M.; Brown, K. L.; Brinkman, F. S.; Hokamp, K.; Potter, A.; Babiuk, L. A.; Griebel, P. J.; Hancock, R. E. W. Bovine and human cathelicidin cationic host defense peptides similarly suppress transcriptional responses to bacterial lipopolysaccharide. *J. Leukoc. Biol.* **2006**, *80* (6), 1563–74.
68. Mookherjee, N.; Lippert, D. N.; Hamill, P.; Falsafi, R.; Nijnik, A.; Kindrachuk, J.; Pistolic, J.; Gardy, J.; Miri, P.; Naseer, M.; Foster, L. J.; Hancock, R. E. W. Intracellular receptor for human host defense peptide LL-37 in monocytes. *J. Immunol.* **2009**, *183* (4), 2688–96.
69. Lau, Y. E.; Rozek, A.; Scott, M. G.; Goosney, D. L.; Davidson, D. J.; Hancock, R. E. W. Interaction and cellular localization of the human host defense peptide LL-37 with lung epithelial cells. *Infect. Immun.* **2005**, *73* (1), 583–91.
70. Yu, H. B.; Kielczewska, A.; Rozek, A.; Takenaka, S.; Li, Y.; Thorson, L.; Hancock, R. E. W.; Guarna, M. M.; North, J. R.; Foster, L. J.; Donini, O.; Finlay, B. B. Sequestosome-1/p62 is the key intracellular target of innate defense regulator peptide. *J. Biol. Chem.* **2009**, *284* (52), 36007–11.
71. De, Y.; Chen, Q.; Schmidt, A. P.; Anderson, G. M.; Wang, J. M.; Wooters, J.; Oppenheim, J. J.; Chertov, O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med.* **2000**, *192* (7), 1069–74.
72. Bandholtz, L.; Ekman, G. J.; Vilhelmsson, M.; Buentke, E.; Agerberth, B.; Scheynius, A.; Gudmundsson, G. H. Antimicrobial peptide LL-37 internalized by immature human dendritic cells alters their phenotype. *Scand. J. Immunol.* **2006**, *63* (6), 410–9.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

73. Hancock, R. E. W.; Rozek, A. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol. Lett.* **2002**, *206* (2), 143–9.

74. Brandenburg, L. O.; Jansen, S.; Wruck, C. J.; Lucius, R.; Pufe, T. Antimicrobial peptide rCRAMP induced glial cell activation through P2Y receptor signalling pathways. *Mol. Immunol.* **2010**, *47* (10), 1905–13.

75. Niyonsaba, F.; Someya, A.; Hirata, M.; Ogawa, H.; Nagaoka, I. Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *Eur J. Immunol.* **2001**, *31* (4), 1066–75.

76. Koczulla, R.; von Degenfeld, G.; Kupatt, C.; Krotz, F.; Zahler, S.; Gloe, T.; Issbrucker, K.; Unterberger, P.; Zaiou, M.; Lebherz, C.; Karl, A.; Raake, P.; Pfosser, A.; Boekstegers, P.; Welsch, U.; Hiemstra, P. S.; Vogelmeier, C.; Gallo, R. L.; Clauss, M.; Bals, R. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J. Clin. Invest.* **2003**, *111* (11), 1665–72.

77. Heilborn, J. D.; Nilsson, M. F.; Kratz, G.; Weber, G.; Sorensen, O.; Borregaard, N.; Stahle-Backdahl, M. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J. Invest. Dermatol.* **2003**, *120* (3), 379–89.

78. Winthrop, K. L. Risk and prevention of tuberculosis and other serious opportunistic infections associated with the inhibition of tumor necrosis factor. *Nat. Clin. Pract. Rheumatol.* **2006**, *2* (11), 602–10.

79. Botsios, C. Safety of tumour necrosis factor and interleukin-1 blocking agents in rheumatic diseases. *Autoimmun. Rev.* **2005**, *4* (3), 162–70.

80. Lillard, J. W., Jr.; Boyaka, P. N.; Chertov, O.; Oppenheim, J. J.; McGhee, J. R. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (2), 651–6.

81. Tani, K.; Murphy, W. J.; Chertov, O.; Salcedo, R.; Koh, C. Y.; Utsunomiya, I.; Funakoshi, S.; Asai, O.; Herrmann, S. H.; Wang, J. M.; Kwak, L. W.; Oppenheim, J. J. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. *Int. Immunol.* **2000**, *12* (5), 691–700.

82. Kovacs-Nolan, J.; Mapletoft, J. W.; Latimer, L.; Babiuk, L. A.; Hurk, S. D. CpG oligonucleotide, host defense peptide and polyphosphazene act synergistically, inducing long-lasting, balanced immune responses in cattle. *Vaccine* **2009**, *27* (14), 2048–54.

83. Kindrachuk, J.; Jenssen, H.; Elliott, M.; Townsend, R.; Nijnik, A.; Lee, S. F.; Gerdts, V.; Babiuk, L. A.; Halperin, S. A.; Hancock, R. E. W. A novel vaccine adjuvant comprised of a synthetic innate defence regulator peptide and CpG oligonucleotide links innate and adaptive immunity. *Vaccine* **2009**, *27* (34), 4662–71.

84. Yeung, A. T.; Gellatly, S. L.; Hancock, R. E. Multifunctional cationic host defence peptides and their clinical applications. *Cell Mol. Life Sci.* **2011**, *68* (13), 2161–76.

85. Hancock, R. E. W. Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.* **2001**, *1* (3), 156–64.

86. McPhee, J. B.; Hancock, R. E. W. Function and therapeutic potential of host defence peptides. *J. Pept. Sci.* **2005**, *11* (11), 677–87.

87. Zhang, H.; Porro, G.; Orzech, N.; Mullen, B.; Liu, M.; Slutsky, A. S. Neutrophil defensins mediate acute inflammatory response and lung dysfunction in dose-related fashion. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2001**, *280* (5), L947–54.

88. Kruse, T.; Kristensen, H. H. Using antimicrobial host defense peptides as anti-infective and immunomodulatory agents. *Expert. Rev. Anti-Infect. Ther.* **2008**, *6* (6), 887–95.

89. Bommarius, B.; Jenssen, H.; Elliott, M.; Kindrachuk, J.; Pasupuleti, M.; Gieren, H.; Jaeger, K. E.; Hancock, R. E. W.; Kalman, D. Cost-effective expression and purification of antimicrobial and host defense peptides in Escherichia coli. *Peptides* **2010**, *31* (11), 1957–65.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch001

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Chapter 2

Structure/Function Link Between Cytokine Domains and Natural and Designed Lytic Peptides: Medical Promise

Jesse M. Jaynes* **and Gregory C. Bernard**

Integrative Biosciences Program, Tuskegee University, Tuskegee, AL 36811
***E-mail: jjaynes@mytu.tuskegee.edu**

Cytokines play key roles in cell-signaling pathways. They are, in effect, the messengers promoting desired interactions by communicating the signals between cells and tissues, thereby causing an adjustment of their behavior ensuring proper temporal and spatial responses. Their actions can be thought of as either autocrine or paracrine acting directly upon the same type of cell that secretes it and/or cells that reside in close proximity. It is known that some cytokines also possess direct antimicrobial activity thus representing an additional component to the evolutionary endowed armamentarium that aids multicellular organisms in their constant battle against pathogens. Ubiquitous smaller natural molecules also exist that exhibit unique antimicrobial activities (lytic peptides/antimicrobial peptides, LPs/AMPs). Recognition of the common structural properties linking distinct cytokine domains to some AMPs (both natural and designed) offers the potential for the production of useful new small molecules to treat a number of disease conditions by modulating imbalances in cellular responses that lead to pathology.

Introduction

Cytokines are types of peptides and proteins that are utilized by organisms as signaling compounds (*1*). The cytokine family consists mainly of smaller water-soluble proteins and glycoproteins with masses between 8 to 30 kilodaltons in size. They are secreted by many types of cells and bind to specific cell-surface

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

receptors (*2*, *3*). Resultant cascades of intracellular signals alter cell function, up-regulate and/or down-regulate genes and result in the production of other proteins including different cytokines. This signaling can also result in an increase in the number of surface receptors for other molecules, or the suppression of the particular cytokine's own effect by feedback-inhibition (*4*). These chemical signals allow one cell to communicate with another. The effect of a particular cytokine on a given cell depends on several factors: (I) the presence/abundance of the complementary receptor(s) on the cell surface, (II) its extracellular abundance and (III) the downstream signals activated by receptor binding; these last two factors are variable and depend upon type (*5*, *6*), and the particular response of the responding cell.

Chemokines are a subset group of small cytokines and are distinguished by their ability to induce directed chemotaxis in responsive cells in close proximity (*7*). Similarities in their structural characteristics are the basis for their classification: small size (all are approximately 8 to 10 kilodaltons in size), and the presence of several cysteine residues in conserved locations that are key to forming their three dimensional shape. Their biological effects are exerted by interaction with transmembrane receptors of the G protein class selectively presented on the surfaces of their target cells. Chemokines released by activated cells form a specific protein concentration gradient (*8*). The concentration gradient so formed is one of the primary metabolic roles played by chemokines that act as guides for the migration and movement of cells to critical areas of the body. Cells that are attracted by chemokines follow a signal of increasing chemokine concentration towards the source of the chemokine production (*9*, *10*).

It is clear from data derived from a great many studies that cytokines, as well as their receptors, play significant roles in the function and development of the innate and adaptive immune responses. Virtually all nucleated cells produce some cytokines. The purpose of which is dependent on the temporal and spatial states of the cells and tissues within the organism (*11*). For the sake of this discussion, below are several cytokines having been selected as examples, emblematic and directly relevant to the main points of this paper.

(A) Interleukin 10 (IL-10) is a pleiotropic cytokine that is produced by monocytes and lymphocytes and plays a role in regulation of the immune system and inflammation. In mice it has been shown that IL-10 is produced by mast cells, the function of which is to impose a block of the inflammatory effect that these cells have at the site of an allergic reaction (*12*). It also seems to reduce inflammation in the bowel and thus may have promise in treating Inflammatory Bowel Disease and Crohn's disease (*13*).

(B) Interleukin 24 (IL-24) is a cytokine that belongs to the IL-10 family and its signals are transduced through two heterodimeric receptors: IL-20R1/IL-20R2 and IL-22R1/IL-20R2. This protein is also known as melanoma-differentiation-associated 7 protein (MDA-7) because of its tumor suppressing activity. Activated monocytes, macrophages and T helper 2 cells produce IL-24 which acts on non-hematopoietic tissues such as skin, lung and reproductive tissues effecting significant roles in wound healing, psoriasis and cancer (*14*, *15*).

(C) Interleukin 8 (IL-8) (C-X-C motif) ligand 8 (CXCL8) is a member of the CXC chemokine family and is one of the major mediators of the inflammatory response. Additionally, it is a potent angiogenic factor. IL-8 is secreted by several cell types including macrophages, epithelial cells and endothelial cells (*16*, *17*). There are more receptors for IL-8 on certain types of cells than most other cytokines; CXCR1 and CXCR2 are the most studied of its receptors (*18*).

(4) Chemokine (C-X-C motif) ligand 10 (CXCL10) or IP-10 is a 10 kDa interferon-gamma-induced protein that is secreted by monocytes, endothelial cells and fibroblasts (*19*). The biological activities of CXCL10 seem to be inhibition of bone marrow colony formation, promotion of T cell adhesion to endothelial cells and anti-tumor activity. CXCL10 is a potent inhibitor of angiogenesis and is thought to respond in an important way to the inflammatory response in the liver and kidney (*20*). Cellular effects are linked through its binding to the surface of CXCR3 receptors (*21*).

(5) Platelet factor 4 (PF4) (C-X-C motif, CXCL4) has been implicated as a regulator of hemostasis by acting as a pro-coagulant; and it has been postulated that it can act oppositely as an anti-coagulant in that it aids in the generation of activated protein C by the thrombin-thrombomodulin complex (*22*). It is a heparin binding chemokine and is known to be capable of inhibiting endothelial cell proliferation and angiogenesis (*23*). Sub-fragments of this protein have been shown to be anti-inflammatory and anti-angiogenic (*24*). Furthermore, CXCL4 causes chemotaxis in monocytes, neutrophils and fibroblasts and elicits this behavior through its interaction with chemokine receptor CXCR3B (*25*).

(6) Chemokine (C-X3-C motif) ligand 1 (CX3CL1) is the only member of the CX3C chemokine family and is also known as human fractalkine (*26*). It is composed of multiple domains with the chemokine type domains presented on top of the outer membrane arrayed protein. There is a soluble 90 kDa derivative of this chemokine that is chemoattractive for T cells and monocytes (*27*). It also promotes the adhesion of leukocytes to activated endothelial cells, doing so by interactions with the singular CX3CR1 receptor (*28*).

The results of numerous studies are supportive of the role of cytokines and chemokines, as well as their receptors, acting as immunoregulators in several human physiological or pathological conditions. It is noteworthy that some of the same cytokines and/or chemokines may play different and, in some cases, opposite functions during the process of cell development and differentiation, or in the context of inflamed tissues in pathological conditions (*29*).

These seemingly conflicting roles of "functional oppositivity" found in many of them can be "structurally" rationalized by recognition that they are comprised of specific and critical domains that exert their activity only under conditions induced by changes in the physiology of the host. Seemingly antithetical properties are only apparent after physical alterations of the molecule have taken place, either through specific protease cleavage of these proteins and/or their assumption of a different conformation, now making "operationally silent" domains "topologically visible" and/or by differential expression of receptors and/or unique receptor combinations that might possess altered binding affinities or specificities.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Overview

Lytic Peptides

Lytic peptides are small proteins that are major components of the antimicrobial defense systems of numerous species (AMPs). They are a ubiquitous feature of nearly all multi-cellular and some single-cellular life forms (*30*). They generally consist of between 10-40 amino acids in length, which have the potential for forming discrete secondary structures. Often, they exhibit the property of amphipathy (Figure 1).

Many of the lytic peptides with capacity to form an α-helix, which have been described in the literature, seem to fall into one of three different classes based, in part, on the arrangement of amphipathy and positive charge density within the molecule (*31*).

1) Melittin (26 amino acids in length and derived from the Honeybee), C-terminal half amphipathic with the N-terminal half primarily hydrophobic (*32*). This peptide's primary role, as a component of bee venom, is protective in the sense of helping to provide part of the "toxicity" in a bee sting.

2) Cecropins (35 amino acids in length and derived from the Giant Silk Moth), N-terminal half amphipathic while the C-terminal half mostly hydrophobic (*33*). These types of peptides were shown to be induced upon bacterial infection of the insect and are a part of their non-humoral anti-prokaryotic immune system.

3) Magainins (23 amino acids in length and derived from the African Clawed Frog), amphipathic the full-length of the molecule (*34*). This peptide is produced in the slimy secretion found on the skin of the amphibian and is primarily a protective compound against infection by prokaryotes.

Conservation of these physical properties is requisite for activity, but the requirements seem to be somewhat nonspecific in terms of amino acid sequence. All classes of lytic peptides differ somewhat in activity (note that Class 3, magainin class, is usually less active in cell membrane disruption than are the other lytic peptide classes) (*35*). A number of highly sequence divergent analogs have been synthesized for each of the peptide classes and some have been found to be substantially more active and less toxic than their natural counterparts (*36*, *37*).

Out work with peptides dates back almost three decades. Initially, our interest lay in producing genetically engineered plants with enhanced disease resistance by the introduction of single genes that encoded proteins or peptides that possess desired antimicrobial activity (*38–40*). Survey of the then current literature yielded a number of papers by Boman, et al. (*41–43*). These papers provided the lab a path

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

to follow. Their critical work outlined the details of the inducible non-humoral immune response in the Giant Silk Moth, their test organism. They found that a suite of proteins was expressed upon infection of the organism by bacteria. One type of small protein, called cecropins, was of interest because they seemed to limit the growth of bacteria by causing their lysis. Since that time, several hundred different natural lytic peptides have been described that demonstrate the ubiquity of these molecules and their success in helping to limit infections in virtually all species (including humans) (*30*). Eventually, a number of designed synthetic peptides were produced and tested utilizing specific design parameters gleaned from years of study.

Working with a number of collaborators, some of the new designs were found to be more active in the lysis of target cells and many were shown to possess a greater breadth of activity (*44*). While the early literature stated that natural lytic peptides possessed only antibacterial activity, we discovered that synthetic designs could be found that eliminated most bacteria, protozoa and fungi and even eliminated cancer cells at very low concentrations, some were even antiviral (*45–57*). Several peptides are now in commercial development and have been shown to confer disease resistance in plant species (when genes encoding these peptides are introduced into plants) (*58–61*), treat various tumors and protect burn-injury patients from infection and promote wound healing (double blind randomized, phase II/III clinical trial for the evaluation of a cream based peptide preparation in the topical treatment of burn wounds conducted at Osmania Hospital in Hyderabad, India. 2008).

In an attempt to understand how these rather small (18 to 23 amino acids in length) and seemingly structurally "uncomplicated" peptides could be effective in eliminating such a wide variety of target cells, a simple way was devised to "view" the lytic peptide structure/function paradigm. Several physical properties of the amino acids were used to design a molecular font called "Molly" (*31*). Molly has been helpful in delineating the clues to lytic peptide structure/function. It also allows for an analysis of other proteins to aid in pattern recognition and correlation of protein function of structurally distinct sequences of related and unrelated proteins.

Molly is representative of the chemical nature of the constitutive amino acids (Figure 2) and was derived by taking the amino acid with the largest 3-dimensional hydrodynamic volume, which is set to 1 (tryptophan) and then making the smaller amino acids proportionately reduced displaying all as circles. Thus, the size of the circle is directly related to amino acid molecular volume (in cubic Angstroms) and, the differences shown among the amino acids, then, are visually depicted. To increase the information of the representation, the hydrophobicity of each amino acid was converted to a color scale (*62*). The most hydrophobic amino acids are the most intense cyan color while those that are less hydrophobic are proportionally less concentrated cyan. Conversely, those amino acids that are the most hydrophilic possess the deepest magenta color. Likewise, a graduated scale of less intense magenta color is used for those amino acids of lower hydrophilic character.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

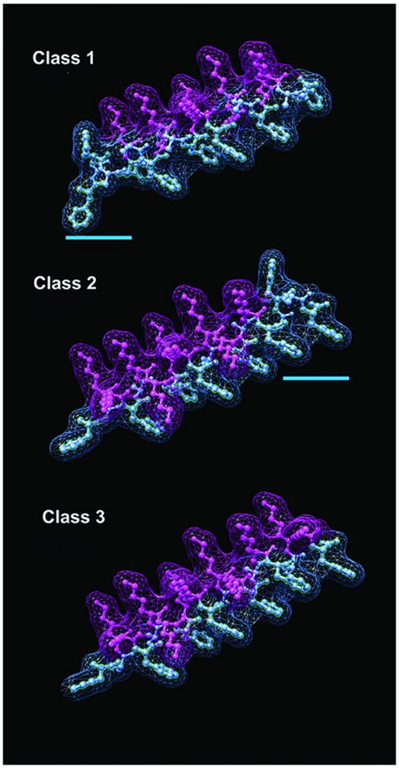


Figure 1. The left side of each molecule indicates the amino-terminus of the peptide while the right side is the carboxy-terminus. The cyan color are regions that are predominately hydrophobic and the magenta color are regions that are hydrophilic. Representative examples of natural peptides that fit this classification system are: melittin-class 1, cecropins-class 2, and magainins-class 3 (many natural peptides fall within this classification system). The blue line indicates the short hydrophibic tail region being amino terminus in class 1 types while carboxy terminus in class 2. Class 3 peptides do not possess a hydrophobic tail. (see color insert)

From this scale, it can be seen that, as amino acids become less hydrophobic or less hydrophilic, they become less pigmented and, therefore, more likely to be “exchangeable” within the protein structure. Also, implicit in this scheme is that, within a particular hue, i.e., amongst hydrophobic amino acids or hydrophilic amino acids, of very similar properties, genetic substitutions would be more likely to occur (generating the variability one observes in proteins of similar function from evolutionarily distant organisms). Of course, changes would be within the specific structural constraints imposed on each particular protein for it to retain its functionality---natural selection, at it again. Most of the amino acid glyphs possess a visual mnemonic symbol that further characterizes their chemical properties. For example, charged amino acids have a “+” or a “-” sign incorporated within their glyph, the thickness of which, is related to the dissociation constant of their ionizing protons; other symbols aid in identifying the rest of the amino acids.

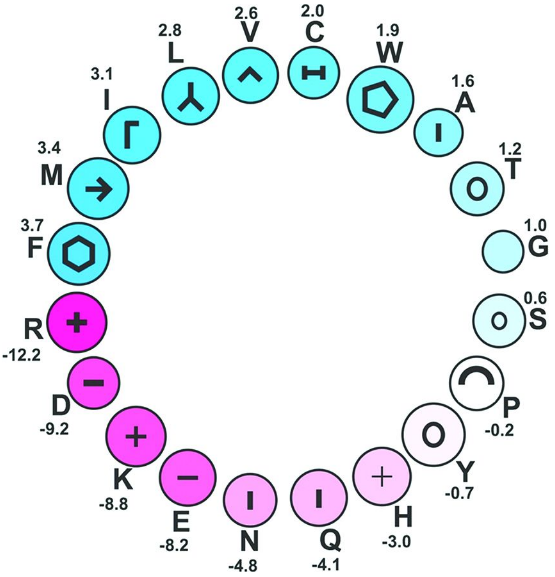


Figure 2. Molly font wheel presented with single letter codes adjacent to each glyph. All hydrophobic amino acids are colored cyan while hydrophilic amino acids are magenta. The number values are relative hydophobicities represented by the number of kcal/mole necessary to exteriorize an amino acid in an α-helix from the inside of a lipid bilayer (62). (see color insert)

It is well documented that lytic peptides demonstrate an ability to efficiently kill many species of bacteria. Interestingly, a number of papers have appeared over the last few years that indicate approximately 30% of cytokines/chemokines also possess direct antimicrobial activity against a number of bacterial pathogens, with modes of action similar to that of lytic peptides (*63–68*). It seems that a few of their domains possess structures similar to the β-defensins. These domains contain several cysteines that lock the peptide in a partial β-sheet conformation that will cause lysis of bacteria under certain conditions (*69–79*). Undoubtedly, this antimicrobial activity plays a significant role in protection of the organism from infection in addition to their many other cellular modulating activities and is another example of the efficiency of design in the evolution of these regulatory proteins.

Anticancer Activity of Lytic Peptides

It was first documented in 1989 that lytic peptides are active in eliminating tumor-derived cells by causing direct osmotic lysis (*80*). Because of this demonstrable activity, it seemed logical that in order to demonstrate in vivo activity the peptide must be injected directly into the tumor. When this is done

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

with just a few injections over a period of several days, tumors are permanently eliminated using the most active anti-tumor peptide, D2A21, yet tested (*81–84*). What happens if this peptide is injected in a site removed from the tumor (in other words, can it express any systemic activity)? The results were unexpected as most of the tumors also disappeared in several animal tumor models (*55*). However, in some cases there was little activity. Why? To determine what might be happening in vivo, radiolabeled D2A21 was chemically synthesized with all alanines labeled with either ^{3}H or ^{14}C. Since the labeling pattern was asymmetric, it enabled us to follow the physical state of the peptide once it had been injected into the animal by comparing the unique ratios of $^{3}H/^{14}C$ that would result if the peptide experienced proteolysis. It was found that within minutes the labeled peptide was hydrolyzed to fragments of various lengths no matter the route of administration but in the circulation approximately 14% of the radiolabel persisted for at least 24 hours with minimal further degradation (unpublished observations). The possibility emerged that the systemic in vivo anti-cancer activity observed was retained within specific fragments of D2A21.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Modulation of Angiogenesis and Inflammation by Lytic Peptide Fragments and Specific Cytokine Domains

Angiogenesis

Angiogenesis is a physiological process that involves the growth of new blood vessels derived from vessels already present. It is a normal process vital for growth and development (*85–88*). However, disease can result from aberrant blood vessel formation and it is a fundamental step in the transition of tumors from the dormant state to one of malignancy (*89*). It has been recognized for many years that in order for tumors to grow beyond a small size an abundance of oxygen and nutrients are requisite for their proliferation. If the vessels feeding the tumor could be eliminated then cancer would become a treatable disease with little chance of a lethal outcome. For the past several years, angiogenesis has been a target for the treatment of cancer and several anti-angiogenic therapies are currently under commercial development (*90*, *91*). A leader of angiogenesis research and the use of anti-angiogenesis therapy as a means to treat cancer was Dr. Judah Folkman (*92–95*). His laboratory's research led to the isolation of several factors that showed significant promise. There was considerable excitement over the therapeutic potential of these naturally derived factors to limit tumor development. Endostatin, a 135 amino acid fragment of collagen XVIII, appeared to hold the most promise. However, because of supply, stability and other issues its development seems to have been slowed (*96*, *97*).

The peptide D2A21's systemic anti-cancer activity in vivo was puzzling. Could this peptide, in addition to promoting lytic effects on cancer cells and tumors, through direct contact, retain anti-angiogenic activity resident somewhere in its structure much like endostatin? Analysis of endostatin was also considered and the protein fragment was viewed in Molly to determine if there were any structural similarities within this molecule and that of D2A21. Figure 3 demonstrates a fragment of endostatin (Endo-f) possesses close physico-chemical relatedness to D2A21 and its 10N fragment.

Endostatin Fragment of Human Collagen XVIII

HSHRDFQPVLHLVALNSPLSGGMRGIRGADFQCFQQARAVGLAGTFRAFLSSRLQ
DLYSIVRRADRAAVPIVNLKDELLFPSWEALFSGSEGPLKPGARIFSFDGKDVLR
HPTWPQKSVWHGSDPNGRRLTESYCETWRTEAPSATGQASSLLGGRLLGQSAASC
HHAYIVLCIENSFMTASK

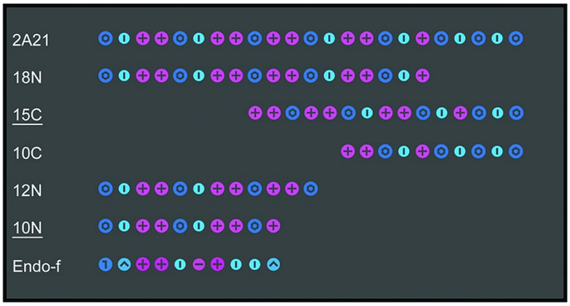


Figure 3. Comparison of an endostatin fragment with full-length D2A21 peptide and aseveral generated fragments displayed in Molly. (see color insert)

In consideration of the above, an initial in vivo experiment was conducted whereby the possible anti-angiogenic behavior of several peptides were evaluated using the well-known technique of implantation into mice of Matrigel deposits impregnated with factors known to stimulate angiogenesis (*98*). Matrigel is a polymeric substance that is relatively inert in animals and can serve as a matrix that allows experimentation in vivo of many different difficult-to-study-processes. Matrigel deposits were surgically implanted on both sides of 4 mice yielding 8 samples per treatment. Prior to implantation, the Matrigel was allowed to imbibe fibroblast growth factor 1 (FGF1). This protein is a powerful inducer of angiogenesis (*94*). Its presence guarantees that sufficient angiogenic activity will be observed within the allotted time period of the experiment. Therefore, any inhibition of angiogenesis is likely to be a real phenomenon as the experiment has been set to heavily favor the angiogenic process. Angiogenesis occurs by day 14 and beginning Day 1 (Day 0 = day of implantation), mice were injected intra-peritoneally daily with about 80 nmoles of the various peptides in 100 µl of normal saline. The animals were sacrificed on Day 14, and each Matrigel deposit divided longitudinally and fixed in 10% buffered formalin. One of the halves of each Matrigel deposit was then sectioned. The sections were histologically analyzed with semi-quantitative/qualitative counting of migration of cells and their subsequent assembly to lumenal structures within the Matrigel.

Analysis of the data demonstrated an approximately equivalent 75% reduction in angiogenesis for both the Endo-f and 10N fragments compared to that observed in the control and other D2A21 peptide fragment treatments. Interestingly, D2A21

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

and other fragments of the peptide showed little reduction in angiogenesis (see Figure 4).

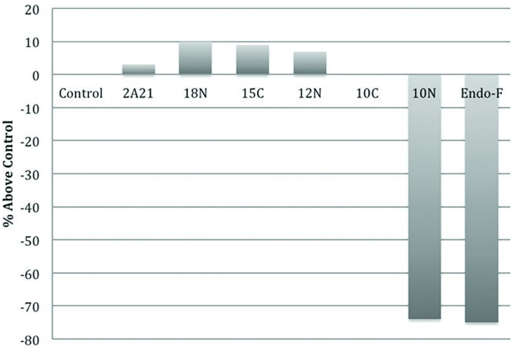


Figure 4. Level of angiogenesis in murine matrigel experiment with control normalized to zero.

Perusal of Figure 3 allows one to compare similarities or differences in the presence or absence of charged amino acids and their position with respect to hydrophobic and other hydrophilic amino acids of the peptides tested in the Matrigel experiment. One can see structural similarities, within sequence motifs, when their glyphs are presented as in the figure.

It is known that endostatin undergoes proteolysis in vivo (*97*). It has been demonstrated that D2A21 experiences a similar fate when injected into mice. Since the "correct" metabolism of these molecules would vary among individual animals, it is not unexpected that some animals would respond better to treatment than others. The yield of potentially therapeutic fragments generated would be entirely at random and could not be expected to be a consistent process, even within one particular animal. The similarities to results, using D2A21 administered from a remote site are striking and are compatible with the occasional variability of an observed anti-tumor effect. At times, and with some animals, the correct processing of D2A21 may be an event of lower probability, yielding a reduced anti-tumor effect. Other animals might process D2A21 in a manner that produces more of the anti-angiogenic fragment hence a greater reduction in disease is observed. Then, the systemic in vivo anti-tumor activity, observed with D2A21, can be accounted for by it's processing, within the animal, to a D2A21-10N-like fragment. The data are also compatible with what has been observed with endostatin---in vivo processing to a "sometimes" anti-angiogenic fragment (unpublished observations).

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Inflammation

Inflammation is a necessary and protective component of an organism's response to negative stimuli such as infections and injury (*99–103*). It is the result of a complex response to these harmful effects. It can be classified as either acute or chronic. Acute being the immediate response of the body to the harmful stimuli and is exemplified by the movement of specific cells to the site of injured tissues resulting in a cascade of biochemical events propagating and maturing the inflammatory response. Chronic inflammation results from the progressive shift of cell types at the site of inflammation resulting in destruction of the inflamed tissue. Many if not all diseases retain inflammation as a significant component of their etiology. Interruption of the chronic inflammatory processes generally results in reduction of pain, continued tissue destruction and is a primary drug development goal.

Since similar effectiveness has been observed from widely divergent disease conditions, it is important to delineate the mechanism of action of the peptide fragments (and selected domains of cytokines and other proteins) as these seemingly different disease conditions must be fundamentally linked physiologically through the commonalities of protein/peptide structure/function. Supportive evidence has been obtained to conclude that one or more of the D2A21 fragments also modulate host-response to certain types of infection and one fragment, in particular, is acting as a potent broadly effective anti-inflammatory agent.

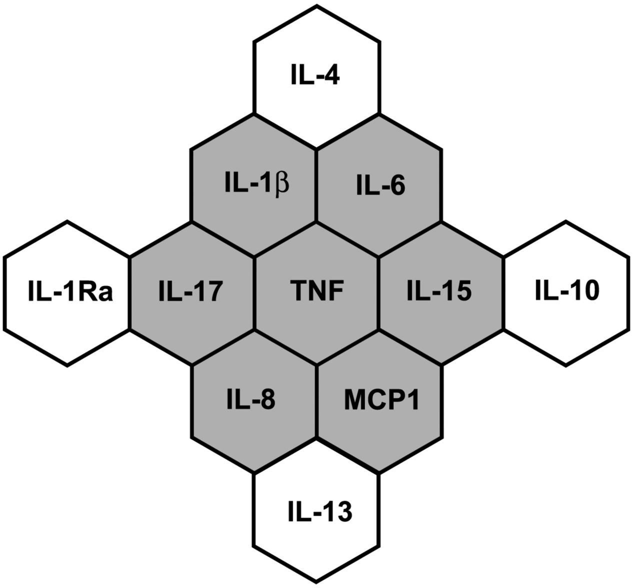
Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

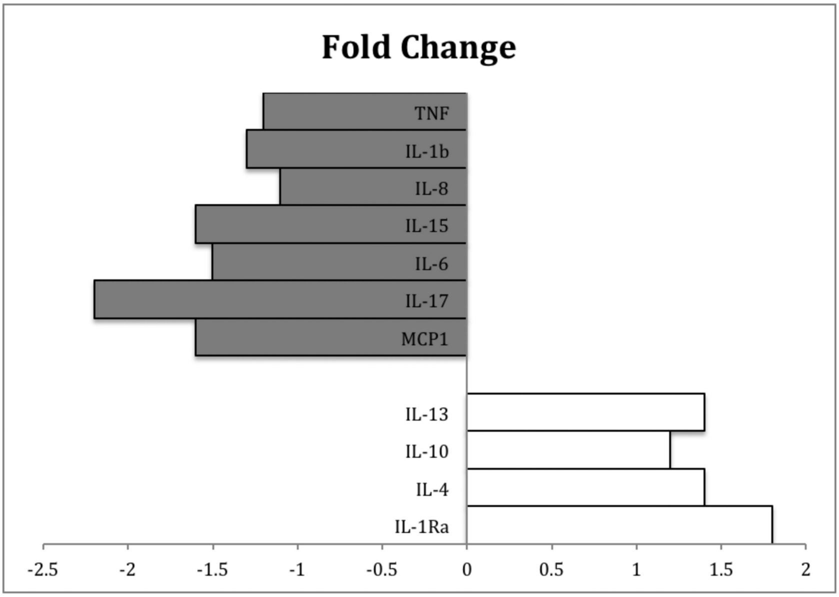
Osteoarthritis

Osteoarthritis (OA) is the most prevalent form of arthritis affecting over 20 million people in the U.S. The progression of disease involves the manifestation of in-joint cartilage lesions by perturbing chondrocyte-matrix associations as a result of alterations of normal cytokine signaling pathways ultimately leading to cartilage degeneration (*105*). During chronic osteoarthritis normal functions of interleukin-1, collagen II, aggrecan synthesis and cell proliferation are reduced and a cytokine imbalance is created between the anti-inflammatory cytokines (IL-4, IL-11, IL-10, IL-13) and soluble tumor necrosis factor receptors which promotes cartilage matrix deterioration and, pro-inflammatory cytokines IL-1, IL-17, IL-6, IL-8, IL-18 and tumor necrosis factor which are involved in cartilage matrix synthesis (*105*).

The unbalanced expression of IL-1 beta, TNF-alpha IL-6, IL-15, IL-17, IL-18, IL-21, leukemia inhibitory factor, and chemokine IL-8 are integral components involved in cartilage matrix degradation and represent important target sites during OA treatment protocols (*106*). IL -1 and TNF-alpha activate matrixmetalloproteinases (specifically MMP-13) which degrade collagens and are found to localize in synovial fluids of OA patients (*105*).

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002


IL-4
IL-1β
IL-6
IL-1Ra
IL-17
TNF
IL-15
IL-10
IL-8
MCP1
IL-13



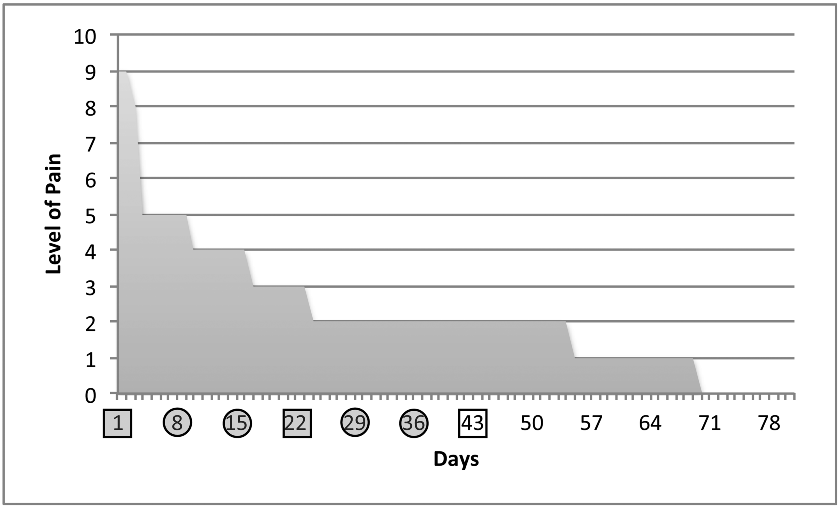


Figure 5. The top panel displays the constellation of factors most involved in the etiology of osteoarthritis. Those considered to be primarily proinflammatory are shaded grey while those thought to be primarily anti-inflammatory are white. The middle panel casts the fold change in these cytokines and bottom panel depicts the numerical evaluation of pain quantitation (107). The shaded squares and circles indicate days when the peptide was administered and the squares are those days in which blood was withdrawn.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Case Study

A human volunteer (JMJ) diagnosed with OA of the right knee joint was treated by subcutaneous injection of the 10N peptide fragment at 0.5 mg/kg body weight once/week for six weeks. To assess the effect of the treatment, blood was drawn prior to, during and after the experiment and then analyzed by Luminex xMap technology. A panel of 39 human cytokines/chemokines was used as an initial screen. Significant changes in the levels of a number of the analytes were observed. Figure 5 depicts the cytokines important in the etiology of OA, the fold changes observed in those cytokines from the test subject's sera. Furthermore, symptoms of pain were quantitated utilizing an accepted numerical pain scale (*107*) and are displayed in figure 5.

From this experiment it can be clearly demonstrated that short duration treatment with 10N significantly reduces pain in a matter of days. Indeed, more than a year later the volunteer remains pain free.

Comparative Structural Analysis of Some Cytokines/Chemokines

Are other natural and synthetic structurally homologous regions similar enough to allow for modulation of angiogenesis? Some possible structural domains found in several cytokines/chemokines can be found in Table I. Innumerable biochemical processes occur at the surfaces of different macromolecules that associate or bind to specific regions on one another within a discrete three-dimensional space. These binding sequences are often rather short stretches of a protein, say, four to eight amino acids in length (*108*).

It is entirely within the realm of possibility that there are only a few amino acids comprising the critical binding region that interacts specifically with target macromolecules initiating an in vivo anti-angiogenic response.

The chemical/structural similarities of 10N with the listed sequences abstracted from some cytokines are relatively easy to recognize. These regions conserve hydrophobicity and charge density to a high degree and their 3-dimensional structure would be quite similar to 10N (see Figure 6(top and bottom), models produced using UCSF Chimera software).

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Table I. Amino acid sequences of selected domains derived from several cytokines, oncostatin and endostatin. All sequences but IDR-1002 (*109*) were obtained from the National Center for Biotechnology Information. Those sequences shaded in grey appear in Figure 6

Designation	*Old Designation*	*Internal Sequence*
CCL5	RANTES	WVREYINSLE
CCL8	MCP-2	WVRDSMKHL
CCL11	Eotaxin	KKWVQDSMK
CCL12	MCP-5	WVKNSINHL
CCL13	MCP-4	WVQNYMKHL
CCL14	CC-1/CC-3	KWVQDYIKDM
CCL15	MIP-5	LTKKGRQVCA
CCL16	---------------	KRVKNAVKY
CCL18	MIP-4	LTKRGRQICA
CCL18	MIP-4	KKWVQKYISD
CCL19	MIP-3 beta	WVERIIQRLQ
CCL20	MIP-3 alpha	IVRLLSKKVK
CCL23	MIP-3	LTKKGRRFC
CCL27	ESkine	LSDKLLRKVI
CCL28	CCK1	VSHHISRRLL

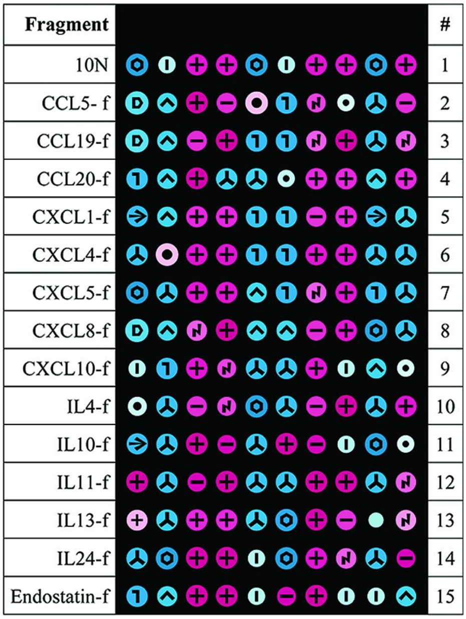
Continued on next page.

Table I. (Continued). Amino acid sequences of selected domains derived from several cytokines, oncostatin and endostatin. All sequences but IDR-1002 (*109*) were obtained from the National Center for Biotechnology Information. Those sequences shaded in grey appear in Figure 6

Designation	*Old Designation*	*Internal Sequence*
XCL2	SCM-1 beta	WVRDVVRSMD
CX3CL1	Fractalkine	WVKDAMQHLD
CXCL1	GRO	MVKKIIEKM
CXCL3	MIP-2 beta	MVQKIIEKIL
CXCL4	PF-4	LYKKIIKKLL
CXCL5	ENA-78	FLKKVIQKIL
CXCL6	GCP-2	FLKKVIQKIL
CXCL7	Pro-platelet pro	IKKIVQKKLA
CXCL8	IL8	WVQRVVEKFL
CXCL10	IP-10	AIKNLLKAVS
CXCL11	IP-9	IIKKVER
CXCL13	B13	WIQRMMEVLR
IL4	----------------	TLENFLERLK
IL5	----------------	TVERLFKNLS
IL7	----------------	FLKRLLQEI
IL10	----------------	MLRDLRDAFS
IL11	----------------	KLDRLLRRLQ
IL13	----------------	HLKKLFRDGQ
IL20	----------------	LLRHLLRL
IL22	----------------	KDTVKKLGE
IL24	----------------	LFRRAFKQLD
IL26	----------------	WIKKLLESSQ
F-LL37	----------------	KRIVQRIKDF
IDR-1002	----------------	VQRWLIVWRIRK
Oncostatin	----------------	SRKGKRLM
Endostatin	F-Collagen XVIII	IVRRADRAAV

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002



Fragment											**#**
10N	⬡	I	⊕	⊕	⬡	I	⊕	⊕	⬡	⊕	1
CCL5- f	D	^	⊕	⊖	○	1	N	⊙	⅄	⊖	2
CCL19-f	D	^	⊖	⊕	1	1	N	⊕	⅄	N	3
CCL20-f	1	^	⊕	⅄	⅄	⊙	⊕	⊕	^	⊕	4
CXCL1-f	→	^	⊕	⊕	1	1	⊖	⊕	→	⅄	5
CXCL4-f	⅄	○	⊕	⊕	1	1	⊕	⊕	⅄	⅄	6
CXCL5-f	⬡	⅄	⊕	⊕	^	1	N	⊕	1	⅄	7
CXCL8-f	D	^	N	⊕	^	^	⊖	⊕	⬡	⅄	8
CXCL10-f	I	1	⊕	N	⅄	⅄	⊕	I	^	⊙	9
IL4-f	○	⅄	⊖	N	⬡	⅄	⊖	⊕	⅄	⊕	10
IL10-f	→	⅄	⊕	⊖	⅄	⊕	⊖	I	⬡	⊙	11
IL11-f	⊕	⅄	⊖	⊕	⅄	⅄	⊕	⊕	⅄	N	12
IL13-f	⊕	⅄	⊕	⊕	⅄	⬡	⊕	⊖	●	N	13
IL24-f	⅄	⬡	⊕	⊕	I	⬡	⊕	N	⅄	⊖	14
Endostatin-f	1	^	⊕	⊕	I	⊖	⊕	I	I	^	15

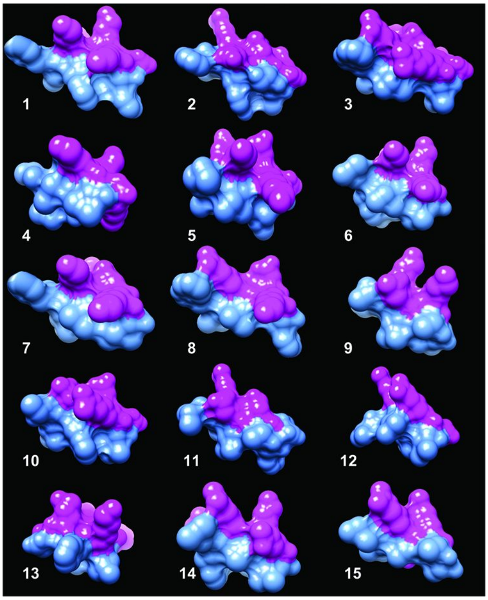


Figure 6. The top panel is a display of selected fragments from several cytokines and endostatin (derivation on the left, designation on the right) compared to 10N. One can easily observe the similarities by viewing the fragments in Molly. The bottom panel displays the three-dimensional representations of the peptide fragments obtained using the UCSF Chimera software. (see color insert)

Within the cytokines and other so noted proteins, by and large, all discrete peptide fragments appear after or between a proline and/or cysteine yielding distinct structural domains. It is predicted that many of the listed sequences would possess anti-angiogenic in vivo activity and possibly anti-inflammatory activity as well, much like 10N (unpublished observations). Thus, the key sequences of each domain, within the specific protein, could function as down-regulators or off/brake switches for cell signaling processes. Indeed, one such fragment from IP-10 has been shown to be a highly effective anti-angiogenic agent in vitro (*104*).

In addition, the screening of peptide phage display libraries has been successfully employed in selecting potential antagonists to several cell signaling membrane receptors such as IL-1R type 1, EGFR and VEGFR (*111–113*).

Postulation

How could the fragments work in this fashion? If cytokines are multi-functional and possess several domains, perhaps their receptors might also possess several binding sites at which to interact and, if propagation of the signal is to proceed, a precise pattern of binding must be initiated between the multi-domain cytokine and it's potentially multi-site receptor (or unique receptors with unique combinations of receptor pairs). Factor in different cytokine/receptor affinities and one can envision a multi-factorial exquisitely balanced control system. Once binding occurs, then the signal is propagated and the cell responds in a predictable and "correct" way or ways. Cytokine/chemokine binding must be consummated for the signal propagation to commence. If either an off switch exists on the receptor or if one of the critical domain-binding sites is occupied signal propagation is halted and if inhibitor binding is particularly robust, it might be some time before the cytokine can interact effectively with its usual target receptor resulting in a lost opportunity for the cell to respond in a timely manner.

In this way, the receptors would be blocked or turned off and then could not transduce the message effectively. Thus, much like a combination keypad, the "correct" binding pattern must be entered to elicit the correct response. If one key is not functional, then the lock cannot be opened. So, the multi-tasking domains of various cytokines will only interact with specific receptors, thereby instituting a measure of control that this complicated multi-dimensional system requires. Depending upon which domains are found in which cytokines different cellular processes will be initiated. So, the various functions: inflammatory or anti-inflammatory, apoptosis or anti-apoptosis, angiogenesis or anti-angiogenesis, turning on sets of genes or turning off sets of genes, etc. all could be controlled in this fashion. Depending upon which "combinations" interact with the receptor keypad and the order in which they are entered, different cellular outcomes are realized. The heterogeneity in the sequences of these proteins may be important to allow some leeway so that alternate "decisions" may be considered. It could be that the di-cation motif of 10N might be more tightly bound and thereby provoke a longer and stronger interaction allowing for the success of the low-dose treatments we have observed in animals and humans (limited anti-inflammatory experiments in achieving positive results in treating IBD, cancer and peripheral neuropathy, manuscripts in preparation). In many of the natural proteins there are also cationic

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

amino acids often adjacent to anionic amino acids that could alter interactions thereby silencing the activity by adjusting binding efficiencies.

Conclusion

It is presumed that many regulatory proteins are constructed with specific and sometimes interchangeable sequences. Selected cytokines have been analyzed and found to possess interesting assemblages of discrete structural domains. In a few cases, when these domains have been "released" from the parent molecule and tested, significant results have been observed (*110*). This mode of protein assembly typifies the "economy of design" that is a hallmark of the natural selection process---the ever-changing shifting results of competing biological necessities combining to make a coherent, functional and beautiful whole. It is emblematic of how evolution has chosen to deal with the formidably difficult, multi-dimensional problems that must be solved for the successful control of the numerous, varied and complex concurrent processes ceaselessly percolating within higher multi-cellular life. By placing critical domains, of opposite function, within many different regulatory proteins (sometimes even the same protein), such as the cytokines, a fine balance between structural specificity and utilitarian redundancy has been achieved. This allows assurance, on average, for speedy, suitable and predictable responses to occur during unforeseen but expected physiological and pathological changes throughout normal growth and development of the individual organism thus ensuring continued species fitness.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

References

1. Gilman A.; Goodman L. S.; Hardman J. G.; Limbird L. E. *Goodman & Gilman's the pharmacological basis of therapeutics*; McGraw-Hill: New York, 2001.
2. Rossi, D.; Zlotnik, A. The biology of chemokines and their receptors. *Annu. Rev. Immunol.* **2000**, *18*, 217–242.
3. Ransohoff, R. M. Chemokines and chemokine receptors: standing at the crossroads of immunobiology and neurobiology. *Immunity* **2009**, *31*, 711–721.
4. Antal, R.; Ulrich, H. A. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu. Rev. Immunol.* **2004**, *22*, 891–928.
5. Stewart, J. Mechanisms of soluble cytokine receptor generation. *J. Immunol.* **2004**, *173*, 5343–5348.
6. Clerici, M. Beyond il-17: new cytokines in the pathogenesis of hiv infection. *Curr. Opin. HIV AIDS* **2010**, *5*, 184–188.
7. Mackay, C. R. Chemokines: immunology's high impact factors. *Nat. Immunol.* **2001**, *2*, 95–101.
8. Gerard, C.; Rollins, B. J. Chemokines and disease. *Nat. Immunol.* **2001**, *2*, 108–15.
9. Poznansky, M. C.; Olszak, I. T.; Foxall, R.; Evans, R. H.; Luster, A. D.; Scadden, D. T. Active movement of T cells away from a chemokine. *Nat. Med.* **2000**, *6*, 543–48.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

10. Förster, R.; Ohl, L.; Henning, G. Lessons learned from lymphocytes: cc chemokine receptor-7 involved in lymphogenic metastasis of melanoma. *J. Natl. Cancer Inst.* **2001**, *93*, 1588–89.
11. Delves P.; Martin S.; Burton D.; Roitt I. *Roitt's Essential Immunology*, 11th ed.; Garland: New York, 2006.
12. Grimbaldeston, M. A.; Nakae, S; Kalesnikoff, J; Tsai, M.; Galli, S. J. Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nat. Immunol.* **2007**, *8*, 1095–104.
13. Braat, H.; Rottiers, P.; Hommes, D. W.; Huyghebaert, N.; Remaut, E.; Remon, J. P.; van Deventer, S. J.; Neirynck, S.; Peppelenbosch, M. P.; Steidler, L. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin. Gastroenterol. Hepatol.* **2006**, *4*, 754–9.
14. Fisher, P. B.; Gopalkrishnan, R. V.; Chada, S.; Ramesh, R.; Grimm, E. A.; Rosenfeld, M. R.; Curiel, D. T.; Dent, P. MDA-7/IL-24, a novel cancer selective apoptosis inducing cytokine gene: from the laboratory into the clinic. *Cancer Biol. Ther.* **2003**, *2*, S23–37.
15. Sauane, M.; Lebedeva, I. V.; Su, Z.; Choo, H.; Randolph, A.; Valerie, K.; Dent, P.; Gopalkrishna, R. V.; Fisher, P. B. Melanoma differentiation associated gene-7/interleukin-24 promotes tumor cell-specific apoptosis through both secretory and nonsecretory pathways. *Cancer Res.* **2004**, *64*, 2988–2993.
16. Smirnova, M. G.; Kiselev, S. L.; Gnuchev, N. V. Role of the pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin-1 beta, interleukin-6 and interleukin-8 in the pathogenesis of the otitis media with effusion. *Eur. Cytokine Netw.* **2003**, *13*, 61–72.
17. Struyf, S.; Proost, P.; Van Damme, J Regulation of the immune response by the interaction of chemokines and proteases. *Adv. Immunol.* **2004**, *81*, 1–44.
18. Brat, D. J.; Bellail, A. C.; Van Meir, E. G. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro-Oncology* **2005**, *7*, 122–33.
19. Neville, L. F.; Mathiak, G.; Bagasra, O The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. *Cytokine Growth Factor Rev.* **1998**, *8*, 207–19.
20. Angiolillo, A. L.; Sgadari, C.; Taub, D. D.; Liao, F.; Farber, J. M.; Maheshwari, S.; Kleinman, H. K.; Reaman, G. H.; Tosato, G. Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. *J. Exp. Med.* **1995**, *182*, 155–62.
21. Clark-Lewis, I.; Mattioli, I.; Gong, J. H.; Loetscher, P. Structure-function relationship between the human chemokine receptor CXCR3 and its ligands. *J. Biol. Chem.* **2003**, *278*, 289–95.
22. Kowalska, M. A.; Mahmud, S. A.; Poncz, M.; Slungaard, A. 2005. Endogenous platelet pf4 promotes in vivo activated protein c (apc) generation and survival after lethal lipopolysaccharide challenge in mice: a potential physiologic role for pf4. *ASH Annu. Meet. Abstr.* **2005**, *106*, 27.
23. Bikfalvi, A.; Gimenez-Gallego, G. The control of angiogenesis and tumor invasion by platelet factor-4 and platelet factor-4-derived molecules. *Semin. Thromb. Hemostasis* **2004**, *30*, 137–44.

24. Maione, T. E.; Gray, G. S.; Petro, J.; Hunt, A. J.; Donner, A. L.; Bauer, S. I.; Carson, H. F.; Sharpe, R. J. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* **1990**, *247*, 77–9.
25. Lasagni, L.; Francalanci, M.; Annunziato, F.; Lazzeri, E.; Giannini, S.; Cosmi, L.; Sagrinati, C.; Mazzinghi, B.; Orlando, C.; Maggi, E.; Marra, F.; Romagnani, S.; Serio, M.; Romagnani, P. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J. Exp. Med.* **2003**, *197*, 1537–49.
26. Sugaya, M.; Nakamura, K.; Mitsui, H.; Takekoshi, T.; Saeki, H.; Tamaki, K. Human keratinocytes express fractalkine/CX3CL1. *J. Derm. Sci.* **2003**, *31*, 179–87.
27. Garton, K. J.; Gough, P. J.; Blobel, C. P.; Murphy, G.; Greaves, D. R.; Dempsey, P. J.; Raines, E. W. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J. Biol. Chem.* **2001**, *276*, 37993–8001.
28. Siafakas, N. M.; Antoniou, K. M.; Tzortzaki, E. G. Role of angiogenesis and vascular remodeling in chronic obstructive pulmonary disease. *Int. J. Chronic Obstruct. Pulm. Dis.* **2007**, *2*, 453–62.
29. Romagnani, P.; Annunziato, F.; Piccinni, M. P.; Maggi, E.; Romagnani, S. Cytokines and chemokines in T lymphopoiesis and T-cell effector function. *Immunol. Today* **2000**, *21*, 416–8.
30. Bechinger, B. Structure and function of membrane-lytic peptides. *Crit. Rev. Plant Sci.* **2004**, *23*, 271–92.
31. Rajasekaran, K.; Jaynes, J. M.; Cary, J. W. Transgenic expression of lytic peptides in food and feed crops to control phytopathogens and preharvest mycotoxin contamination mycotoxin prevention and control in agriculture. ACS Symposium Series 1031; American Chemical Society: Washington, DC, 2010, pp 119−42.
32. Haberman, E. Bee and wasp venoms. *Science* **1972**, *177*, 314–22.
33. Hultmark, D.; Steiner, H.; Rasmuson, T.; Boman, H. G. Purification and properties of three inducible bacterial proteins from the hemolymph of immunized pupae of Hyalophora cecropia. *Eur. J. Biochem.* **1980**, *106*, 7–16.
34. Zasloff, M. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449–53.
35. Kelly, D.; Wolters, W.; Jaynes, J. M. The effect of lytic peptides on fish-pathogenic bacteria. *J. Fish Dis.* **1990**, *13*, 317–21.
36. Gruzensky, W.; Mannis, M. J.; Schwab, I. R.; Jaynes, J. The use of biotechnology a cecropin analog, hecate, against acanthamoeba in-vitro. *Invest. Ophthalmol. Visual Sci.* **1994**, *35*, 1337.
37. Gunshefski, L.; Mannis, M. J.; Cullor, J. S.; Schwab, I. R.; Jaynes, J; Smith, W. L.; Mabry, E.; Murphy, C. J. In vitro antimicrobial activity of shiva-11 against ocular pathogens. *Cornea* **1994**, *13*, 237–42.
38. Cetiner, S.; Jaynes, J. M.; Blackmon, B. Effect of novel lytic peptides on plant pathogenic fungi. *Hortscience* **1987**, *22*, 1057.
39. Destefano-beltran, L.; Jaynes, J. M.; Clark, C. Effect of novel lytic peptides on plant pathogenic bacteria. *Phytopathology* **1987**, *77*, 1768.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

40. Jaynes, J. M.; Xanthopoulos, D.; Destefano-Beltran, L.; Dodds, J. H. Increasing bacterial disease resistance in plants utilyzing antibacterial genes from insects. *Bioessays* **1987**, *6*, 263–70.
41. Boman, H. G.; Kockum, K.; Lee, J; Xanthopoulos, K. G.; Bennich, H.; Engstrom, A.; Merrifield, B.; Andreu, D. On The primary structure of lysozyme, cecropins and attacins from Hyalophora cecropia. *Dev. Comp. Immun.* **1985**, *9*, 551–58.
42. Hultmark, D.; Engstrom, A.; Andersson, K.; Steiner, H.; Bennich, H.; Boman, H. G. Insect immunity: attacins, a family of antibacterial proteins from Hyalophora cecropia. *EMBO J* **1983**, *2*, 571–76.
43. Hofsten, P.; Faye, I.; Kockum, K.; Lee, J.; Xanthopoulo, K. S. G.; Boman, L. A.; Boman, H. G.; Engstrom, A.; Andreu, D.; Merrifield, B. Molecular cloning, cdna sequencing, and Chemical synthesis of cecropin b from Hyalophora cecropia. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2240–243.
44. Jaynes, J. M. Lytic peptides: harbingers of a new age in the treatment of disease. *New Sci* **1989**, 42–44.
45. Schwab, U.; Gilligan, P.; Jaynes, J.; Henke, D. In vitro acitivities of designed antimicrobial peptides against multidrug-resistant cystic fibrosis pathogens. *Antimicrob. Agents Chemother.* **1999**, *43*, 1435–1440.
46. Arrowood, M. J.; Jaynes, J. M.; Healy, M. C. In vitro activities of lytic peptides against the sporozoites of cryptosporidium parvum. *Antimicrob. Agents Chemother.* **1991**, *35*, 224–27.
47. Miller, M. A.; Garry, R. F.; Jaynes, J. M.; Montelaro, R. C. A structural correlation between lentivirus transmembrane proteins and natural cytolytic peptides. *AIDS Res. Hum. Retroviruses* **1991**, *7*, 511–19.
48. van der Biezen, E. A. Quest for antimicrobial genes to engineer disease-resistant crops. *Trends Plant Sci.* **2001**, *6*, 89–91.
49. Jaynes, J. M.; Nagpala, P.; Destefano-Beltran, L.; Huang, J. H.; Kim, J. H.; Denny, T.; Cetiner, S. Expression of a cecropin b lytic peptide in transgenic tobacco confers enhanced resistance to bacterial wilt caused by Pseudomonas solanacearum. *Plant Sci.* **1993**, *89*, 43–53.
50. Jung, Y. I.; Seo, H.; Yang, M.; Robb, E.; Nazar, R.; Lee, S. Plant defense gene promoter enhances the reliability of shiva-1 gene-induced resistance to soft rot disease in potato. *Planta* **2004**, *220*, 165–71.
51. Barr, S. C.; Rose, D.; Jaynes, J. M. Activity of lytic peptides against intracellular Trypanosoma cruzi amastigotes in vitro and parasitemias in mice. *J. Parasitol.* **1995**, *81*, 974–78.
52. Baghian, A.; Jaynes, J.; Enright, F. An amphipathic alpha-helical synthetic peptide analogue of melittin inhibits herpes simplex virus-1 (hsv-1) induced cell fusion and virus spread. *Peptides* **1997**, *18*, 177–183.
53. Martin, A.; Danforth, H. D.; Jaynes, J. M.; Thornton, J. Evaluation of the effect of peptidyl membrane-interactive molecules on avian coccidia. *Parasitol Res* **1999**, *85*, 331–36.
54. Lushbaugh, W. B.; Paxton, A.; Shah, P. A.; Banga, A.; Jaynes, J.; Cleary, J.; Finley, R. W. Use of intravaginal microbicides to prevent acquisiton of trichomonas vaginalis infection in lactobacillis-pretreated, estrogenized young mice. *Amer. J. Trop. Med. Hyg.* **2000**, *63*, 284–89.

55. Arlotti, J. A.; Cimino, T. S.; Nguyen, T. S.; Dhir, R.; Thomas, A.; Jaynes, J. M.; Caldwell, A. L.; Getzenberg, R. H. Efficacy of a synthetic lytic peptide in the treatment of prostate cancer. *Urol. Onccol.* **2001**, *6*, 97–102.
56. Ballweber, L. M.; Jaynes, J. M.; Stamm, W. E.; Lampe, M. F. In vitro microbicidal activity of cecropin peptides d2a21 and d4e1 and gel formulations containing 0.1-2.0% d2a21 against Chlamydia trachomatis. *Antimicrob. Agents Chemother.* **2002**, *46*, 34–41.
57. Ma, J.; Kennedy-Stoskopf, S.; Jaynes, J.; Tompkins, W. Inhibitory Activity of Synthetic Peptide Antibiotics on Feline Immunodeficiency Virus In Vitro. *J. Virol* **2002**, *76*, 9952–9961.
58. Jaynes, J. M.; Xanthopoulos, D.; Destefano-Beltran, L.; Dodds, J. H. Increasing bacterial disease resistance in plants utilyzing antibacterial genes from insects. *BioEssays* **1987**, *6*, 263–70.
59. Jaynes, J. M.; Hammerschlag, F. A.; Zimmerman, L. H.; Owens, L. D. Use of genes encoding novel lytic peptides and proteins that enhance microbial disease resitance in plants. *Acta Horticulturae, Second International Symposium on In Vitro Culture and Horticultural Breeding* **1993**, *336*, 33–39.
60. Cary, J. W.; Rajasekaran, K.; Jaynes, J. M.; Cleveland, T. E. Transgenic expression of a gene encoding a synthetic antimicrobial peptide results in inhibition of fungal growth in vitro and in planta. *Plant Sci.* **2000**, *154*, 171–81.
61. Rajasekaran, K.; Cary, J. W.; Jaynes, J. M.; Cleveland, T. E. Disease resistance conferred by the expression of a gene encoding a synthetic peptide in transgenic cotton (Gossypium hirsutum l.) plants. *Plant Biotechnol. J.* **2005**, *3*, 545–54.
62. Engelman, D. M.; Steitz, T. A.; Goldman, A. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Bioeng.* **1986**, *15*, 321–53.
63. Linge, H. M.; Collin, M.; Nordenfelt, P.; Mörgelin, M.; Malmsten, M.; Egesten, A. The human CXC chemokine granulocyte chemotactic protein 2 (GCP-2)/CXCL6 possesses membrane-disrupting properties and is antibacterial. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16879–84.
64. Nordahl, E. A.; Rydengård, V.; Nyberg, P.; Nitsche, D. P.; Mörgelin, M.; Malmsten, M.; Björck, L.; Schmidtchen, A. Activation of the complement system generates antibacterial peptides. *Antimicrob. Agents Chemother.* **2006**, *50*, 3786–92.
65. Xiong, Y. Q.; Bayer, A. S.; Elazegui, L.; Yeaman, M. R. A synthetic congener modeled on a microbicidal domain of thrombin- induced platelet microbicidal protein 1 recapitulates staphylococcidal mechanisms of the native molecule. *J. Immunol.* **2008**, *180*, 3366–74.
66. Björstad, A.; Fu, H.; Karlsson, A.; Dahlgren, C.; Bylund, J Interleukin-8-derived peptide has antibacterial activity. *Int. Immunol.* **2007**, *19*, 1095–102.
67. Shelkine, Y.; Sirsjo, A. CXCL16 is a novel mediator of the innate immunity of epidermal keratinocytes. *Atherosclerosis* **2008**, *197*, 487–95.
68. Maerki, C.; Meuter, S.; Liebi, M.; Mühlemann, K.; Frederick, M. J.; Yawalkar, N.; Moser, B.; Wolf, M. Potent and broad-spectrum antimicrobial activity of CXCL14 suggests an immediate role in skin infections. *Antimicrob. Agents Chemother.* **2008**, *52*, 2599–607.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

69. Zou, G.; de Leeuw, E.; Li, C.; Pazgier, M.; Li, C.; Zeng, P.; Lu, W. Y.; Lubkowski, J.; Lu, W. Toward understanding the cationicity of defensins Arg and Lys versus their noncoded analogs. *J. Biol. Chem.* **2007**, *282*, 1819–29.
70. Pazgier, M.; Prahl, A.; Hoover, D. M.; Lubkowski, J. Studies of the biological properties of human beta-defensin 1. *J. Leukocyte Biol.* **2005**, *77*, 652–60.
71. Oliaro, J.; Dudal, S.; Liautard, J.; Andrault, J. B.; Liautard, J. P.; Lafont, V. gamma9Vdelta2 T cells use a combination of mechanisms to limit the spread of the pathogenic bacteria Brucella. *J. Immunol.* **2006**, *176*, 544–56.
72. Schaffner, A.; King, C. C.; Schaer, D.; Guiney, D. G. Induction and antimicrobial activity of platelet basic protein derivatives in human monocytes. *Angiogenesis* **2001**, *4*, 45–51.
73. Mayo, K. H.; van der Schaft, D. W.; Griffioen, A. W. Designed beta-sheet peptides that inhibit proliferation and induce apoptosis in endothelial cells. *Biopolymers* **2009**, *91*, 1–13.
74. Bourbigot, S.; Dodd, E.; Horwood, C.; Cumby, N.; Fardy, L.; Welch, W. H.; Ramjan, Z.; Sharma, S.; Waring, A. J.; Yeaman, M. R.; Booth, V. Antimicrobial peptide RP-1 structure and interactions with anionic versus zwitterionic micelles. *Microbiology* **2007**. *153*, 1187−97.
75. Mukhopadhyay, K.; Whitmire, W.; Xiong, Y. Q.; Molden, J.; Jones, T.; Peschel, A.; Staubitz, P.; Adler-Moore, J.; McNamara, P. J.; Proctor, R. A.; Yeaman, M. R.; Bayer, A. S. In vitro susceptibility of Staphylococcus aureus to thrombin-induced platelet microbicidal protein-1 (tPMP-1) is influenced by cell membrane phospholipid composition and asymmetry. *Antimicrob. Agents Chemother.* **2004**, *48*, 4395–404.
76. Yount, N. Y.; Gank, K. D.; Xiong, Y. Q.; Bayer, A. S.; Pender, T.; Welch, W. H.; Yeaman, M. R. Platelet microbicidal protein 1: structural themes of a multifunctional antimicrobial peptide. *J. Biol. Chem.* **2004**, *279*, 35849–57.
77. Schröder, J. M.; Harder, J. Antimicrobial skin peptides and proteins. *Cell Mol. Life Sci* **2006**, *63*, 469–86.
78. Bevins, C. L. Paneth cell defensins: key effector molecules of innate immunity. *Biochem. Soc. Trans.* **2006**, *34*, 263–66.
79. Hirsch, T.; Metzig, M.; Niederbichler, A.; Steinau, H. U; Eriksson, E.; Steinstraesser, L. Role of host defense peptides of the innate immune response in sepsis: host defense peptides in wound healing. *J. Immunol.* **2007**, *179*, 7684–91.
80. Jaynes, J. M.; Jeffers, G. W.; Julian, G. R.; White, K. L.; Enright, F. M. in vitro cytocidal effect of lytic peptides on several transformed mammalian cell lines. *Peptide Res.* **1989**, *2*, 157–60.
81. Robertson, C.; Roberson, K.; Pinero, A.; Jaynes, J.; Paulson, D. Peptidyl membrane-interactive molecules are cytotoxic to prostate cancer cells in vitro. *World J. Urol.* **1998**, *16*, 405–09.
82. Makhuli, M. J.; Roberson, K. M.; Poulton, S. H. M.; Vollmer, R. T.; Padilla, G. M.; Edwards, D. W.; Yancey, D. R.; Caldwell, A. L.; Jaynes, J. M.; Robertson, C. N. In vivo, synthetic lytic peptides exhibit anti-tumor activity against prostate cancer. *American Urological Annual Meeting, Honolulu, HI* **1998**.

83. Papo, S; Eisenbach, L; Shai, Y A novel lytic peptide composed of dl-amino acids selectively kills cancer cells in culture and in mice. *J. Biol. Chem.* **2003**, *278*, 21018–23.
84. Lehmann, J.; Retz, M.; Sidhu, S.; Sutthman, H.; Sell, M.; Paulsen, F.; Harder, J.; Unteregger, G.; Stockle, M. Antitumor activity of the antimicrobial peptide magainin II against bladder cancer cell lines. *Eur. Urol.* **2006**, *50*, 141–47.
85. Folkman, J.; Klagsbrun, M. Angiogenic factors. *Science* **1987**, *235*, 442–7.
86. Freedman, S. B.; Isner, J. M. Therapeutic angiogenesis for ischemic cardiovascular disease. *J. Mol Cell Cardiol.* **2001**, *33*, 379–93.
87. Stegmann, T. J.; Hoppert, T.; Schneider, A.; Gemeinhardt, S.; Köcher, M.; Ibing, R.; Strupp, G. Induction of myocardial neoangiogenesis by human growth factors. A new therapeutic approach in coronary heart disease. *Herz* **2000**, *25* (6), 589–99.
88. Folkman, J. Angiogenic therapy of the human heart. *Circulation* **1998**, *97*, 628–9.
89. Folkman, J. Fighting cancer by attacking its blood supply. *Sci. Amer.* **1996**, *275* (3), 150–4.
90. Entremed. 2002. Safety and efficacy study of rhangiostatin administered in combination with paclitaxel and carboplatin to patients with non-small-cell lung cancer, clinicaltrials.gov.
91. Dana-Farber Cancer Institute. 2000. Boston hospitals report early results of endostatin clinical trial. Press release.
92. Folkman, J. Tumor angiogenesis: Therapeutic implications. *N. Engl. J. Med.* **1971**, *285*, 1182–86.
93. Cavallo, T.; Sade, R.; Folkman, J.; Cotran, R. S. Tumor angiogenesis: rapid induction of endothelial mitoses demonstrated by autoradiography. *J. Cell Biol.* **1972**, *54*, 408–20.
94. Folkman, J. Angiogenesis: an organizing principle for drug discovery. *Nat. Rev. Drug Discov* **ery2007**, *6*, 273–86.
95. Folkman, J. Antiangiogenesis in cancer therapy--endostatin and its mechanisms of action. *Exp. Cell. Res.* **2006**, *312*, 594–07.
96. Kulke, M. H.; Bergsland, E. K.; Ryan, D. P.; Enzinger, P. C.; Lynch, T. J.; Zhu, A. X.; Meyerhardt, J. A.; Heymach, J. V.; Fogler, W. E.; Sidor, C.; Michelini, A.; Kinsella, K.; Venook, A. P.; Fuchs, C. S. Phase II study of recombinant human endostatin in patients with advanced neuroendocrine tumors. *J. Clin. Oncol.* **2006**, *22*, 3555–61.
97. Holaday, J. W.; Berkowitz, B. A. Antiangiogenic drugs: insights into drug development from endostatin, avastin and thalidomide. Mol Interventions **2009**, *9*, 157−166
98. Kleinman, H. K.; Martin, G. R. Matrigel: basement membrane matrix with biological activity. *Semin. Cancer Biol.* **2005**, *15*, 378–86.
99. Ferrero-Miliani, L.; Nielsen, O. H.; Andersen, P. S.; Girardin, S. E. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. *Clin. Exp. Immunol.* **2007**, *147*, 227–235.
100. Abbas, A. B.; Lichtman, A. H. *Basic Immunology. Functions and disorders of the immune system*, 3rd ed.; Saunders: Philadelphia, PA, 2009; Chapter 2 Innate Immunity.
101. Coussens, L. M.; Werb, Z. Inflammation and cancer. *Nature* **2002**, *420*, 860–867.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

102. Eming, S. A.; Krieg, T; Davidson, JM Inflammation in wound repair: molecular and cellular mechanisms. *J. Invest. Dermatol.* **2007**, *127*, 514–525.

103. Serhan, C. N. Controlling the resolution of acute inflammation: a new genus of dual anti-inflammatory and pro-resolving mediators. *J. Periodontol.* **2008**, *79*, 1520–1526.

104. Yates, C. C., Rogers, M., Jaynes, J., Wells, A., Bodnar, R. J., Turner T.. 2011. IP-10 fragment is the functional motif that blocks endothelial cell motility and vessel formation. Accepted in *PLoS One*.

105. Goldring, M. Osteoarthritis and cartilage: the role of cytokines. *Curr. Rhemumatol. Rep.* **2000**, *2*, 459–465.

106. Kapoor, M.; Martel-Pelletier, J.; Lajeunesse, D.; Pelletier, J.-P.; Fahmi, H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat. Rev. Rheumatol.* **2011**, *7*, 33–42.

107. McCaffery, M.; Pasero, C. *Pain: Clinical Manual*, 2nd ed.; Mosby: St. Louis, MO, 1999.

108. Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular biology of the cell*, 5th ed.; Garland Science: New York, 2007.

109. Nijnik, A.; Madera, L.; Ma, S.; Waldbrook, M.; Elliott, M. R.; Easton, D. M.; Mayer, M. L.; Mullaly, S. C.; Kindrachuk, J.; Jenssen, H.; Hancock, R. E. W. Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *J. Immunol.* **2010**, *184*, 2539–2550.

110. Manuscript in preparation

111. Gram, H.; Strittmatter, U.; Lorenz, M.; Gluck, D.; Zenke, G. Phage display as a rapid gene expression system: production of bioactive cytokine-phage and generation of neutralizing monoclonal antibodies. *J. Immunol. Methods* **1993**, *161*, 169–176.

112. Bass, S.; Greene, R.; Wells, J. A. Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins* **1995**, *8*, 309–314.

113. Molek, P.; Strukelj, B.; Bratkovic, T. Peptide Phage Display as a Tool for Drug Discovery: Targeting Membrane Receptors. *Molecules* **2011**, *16*, 857–887.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch002

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Chapter 3

The Potential of Frog Skin Antimicrobial Peptides for Development into Therapeutically Valuable Anti-Infective Agents

J. M. Conlon*

**Department of Biochemistry, Faculty of Medicine and Health Sciences,
United Arab Emirates University, 17666 Al Ain, U.A.E.
*E-mail: jmconlon@uaeu.ac.ae**

Granular glands in the skins of several frog species synthesize peptides with broad spectrum antimicrobial activity against bacteria and fungi that show promise for use against antibiotic-resistant strains of pathogenic microorganisms. However, their therapeutic potential is limited by their varying degrees of cytotoxicity towards mammalian cells, such as human erythrocytes. This review assesses potential clinical applications of a range of frog skin antimicrobial peptides for use against multidrug-resistant microorganisms, such as the Gram-negative bacteria *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*; the Gram-positive bacterium *Staphylococcus aureus*; and the opportunistic yeast pathogens *Candida* spp. Analogs of the naturally occurring peptides containing one or more amino acid substitutions have been developed that retain high antimicrobial potency but are non-hemolytic. Treatment and prevention of *acne vulgaris* and periodontal disease are identified as areas in which frog skin antimicrobial peptides might find future applications.

Introduction

The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics constitutes a serious threat to public health and has necessitated a search for novel types of antimicrobial

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

agent to which the microorganisms have not been exposed (*1*). Although effective new types of antibiotics against multidrug-resistant Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) have been introduced or are in clinical trials, the situation regarding new treatment options for infections produced by multidrug-resistant Gram-negative pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Stenotrophomonas maltophilia* is less encouraging (*2*). There is an urgent need for new types of antimicrobial agents with activity against these microorganisms that also possess appropriate pharmacokinetic and toxicological profiles.

Peptides with potent antibacterial and antifungal activity play an important role in the system of innate immunity that predates adaptive immunity and constitutes the first-line defense against invading pathogens for a wide range of vertebrate and invertebrate species (*3*). Anti-infective compounds based upon such peptides are being increasingly considered as potential therapeutic agents (*4*). Although development of resistance to antimicrobial peptides has been demonstrated experimentally *in vitro* (*5*), it occurs at rates that are orders of magnitude lower than those observed for conventional antibiotics. Major obstacles to the development of peptide-based anti-infective drugs, particularly if they are to be administered systemically, are their cytolytic activities towards human cells such as erythrocytes, hepato-and nephro-toxicities *in vivo*, their short half-lives in the circulation, and possible immunogenicities (*6*). However, peptides applied to infected skin or skin lesions in the form of sprays or ointments can penetrate into the *stratum corneum* to kill microorganisms so that future therapeutic applications are more likely to involve topical rather than systemic administration.

Skin secretions from many species of Anura (frogs and toads) contain a wide range of compounds with biological activity, often in very high concentration, that have excited interest because of their potential for drug development (*7*). Among these substances are host-defense peptides with broad-spectrum antibacterial and antifungal activities and the ability to permeabilize mammalian cells (*8*). Over 20 years have passed since the discovery of the magainins in the skin of African clawed frog, *Xenopus laevis*. These peptides, identified independently by Michael Zasloff at the National Institutes of Health, Bethesda, U.S.A. (*9*) and by the group of Dudley H. Williams at the University of Cambridge, U.K. (*10*), were the first amphibian peptides with antimicrobial activity to be fully characterized. Since that time several hundred such peptides have been isolated from the skin secretions of many other frogs belonging to different families.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Molecular Properties of Frog Skin Antimicrobial Peptides

Frog skin antimicrobial peptides vary in size from as small as 8 up to 48 amino acid residues (*11*) and a comparison of their amino acid sequences reveals the lack of any conserved domains that are associated with biological activity. However, with few exceptions, these peptides are cationic, generally with a molecular charge between +2 and +6 at pH 7 due to the presence of multiple lysine residues, and contain at least 50% hydrophobic amino acids of which leucine and isoleucine are usually the most abundant. Circular dichroism and NMR studies have shown that they generally lack stable secondary structure in aqueous solutions but have the propensity to form an amphipathic α-helix in the environment of a phospholipid vesicle or in a membrane-mimetic solvent such as 50% trifluoroethanol-water (*12*). There is no single mechanism by which peptides produce cell death but their action does not involve binding to a specific receptor rather a non-specific interaction with the bacterial cell membrane that results in permeabilization and ultimate disintegration (*13*). Consequently, the frog skin peptides are usually active against microorganisms that are resistant to currently licensed antibiotics due to their markedly different and highly destructive mode of action.

The frog skin antimicrobial peptides may be grouped together in peptide families on the basis of limited similarities in amino acid sequence. Skin secretions from a single species frequently contain several members of a particular family that are presumed to have arisen from multiple duplications of an ancestral gene. The molecular heterogeneity of the peptides within a particular family is considerable and this variation in primary structure is reflected in a wide variability in antimicrobial potencies and specificities for different microorganisms (*14*). It has been suggested that this multiplicity may provide a broader spectrum of defense against the range of pathogenic microorganisms encountered in the environment (*15*) but conclusive evidence to support this assertion is still required.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Development of Potent, Nontoxic Anti-Infective Agents Based upon Frog Skin Peptides

Despite showing potent activity against strains of antibiotic-resistant bacteria and against certain pathogenic fungi and protozoa, the potential of frog skin peptides as therapeutic agents has not been realized. No anti-infective peptide based upon their structures has yet been adopted in clinical practice. This review will examine possible clinical application of several well characterized antimicrobial peptides that have been isolated from frog skin. Examples of such peptides with therapeutic potential are shown in Table 1.

The value of many of these peptides is limited by their potent cytotoxic activity against mammalian cells such as human erythrocytes. The antimicrobial activities of α-helical peptides against microorganisms and their cytotoxicities against mammalian cells are determined by complex interactions between cationicity, hydrophobicity, conformation (α-helicity) and amphipathicity (*12*, *13*). These are not independent variables so that an amino acid substitution will generally affect more than one parameter simultaneously.

The bacterial cytoplasmic cell membrane is rich in anionic phospholipids and negatively charged lipopolysaccharides whereas the plasma membrane of mammalian cells contains a much higher proportion of zwitterionic phospholipids together with uncharged cholesterol and cholesterol esters. Structure-activity studies with a range of naturally occurring and model peptides [reviewed in (*16*)] have shown that that an increase in peptide cationicity promotes interaction with the negatively charged bacterial cell membrane and so increases antimicrobial potency whereas increasing overall hydrophobicity, helicity, and amphipathicity generally enhances hemolytic activity. Table 2 provides examples of naturally occurring frog skin antimicrobial peptides with relatively high hemolytic activity that have been transformed into analogs that retain potency against pathogenic microorganisms but show appreciably reduced toxicity against human erythrocytes. Increase in cationicity has been effected by replacement of appropriate substitutions of amino acids on the hydrophilic face of the helix by L-lysine. This strategy was employed successfully to transform magainin-2 into the potent broad-spectrum analog, pexiganan (MSI-78). Pexiganan contains an additional five lysyl residues and an α-amidated C-terminus and showed potential for treatment of infected foot ulcers in diabetic patients (*17*). However, L-lysine is readily incorporated into an α-helical structure so that substitution by this amino acid may result in increased helicity concomitant with increased cationicity. Table 2 provides examples of potentially therapeutically valuable analogs that contain D-lysine substitutions and so show increased cationicity concomitant with a less stable α-helical conformation.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Temporin-DRa

The dodecapeptide temporin-DRa was first isolated from skin secretions of the California red-legged frog *Rana draytonii* (*18*) and shows potential for treatment of infections produced by methicillin-resistant strains of *Staphylococcus aureus* (MRSA). Methicillin resistance first appeared among nosocomial isolates of *S. aureus* in 1961 and since that time MRSA has emerged to become a major phenotype in hospitals worldwide with a high rate of mortality (*19*). MRSA produces an alternative transpeptidase with low affinity for β-lactam antibiotics which results in not only methicillin resistance but *in vivo* non-susceptibility to almost all β-lactam antibiotics. More recently, new strains of MRSA have emerged in the community causing infections in young, otherwise healthy people (*20*). In addition to β-lactam resistance, MRSA strains may exhibit multidrug resistance, including non-susceptibility to several other classes of antibiotics such as quinolones, macrolides and sulphonamides (*21*).

Temporin-DRa shows high growth-inhibitory potency against clinical isolates of MRSA (Minimum Inhibitory Concentration, MIC = 8 μM) and has the advantages of ease of synthesis and high solubility (*16*). Its therapeutic potency is limited by moderately high hemolytic activity (LC_{50} = 65 μM). However, the analog containing the amino acid substitution Val^7 → L-Lys and the analogs containing the helix-destabilizing substitutions Thr^5→ D-Lys and Asn^8 → D-Lys retain activity against MRSA (MIC in the range 8 – 16 μM) but have very low hemolytic activity (LC_{50} > 300 μM) (*16*) (Figure 1). In contrast, analogs containing the substitutions Gly^4 → L-Lys and Asn^8 → L-Lys show increased potency (2-fold) against MRSA but also a 2-fold increase in hemolytic activity. As well as increasing cationicity, the substitution Val^7→ Lys decreases amphipathicity by increasing the polar angle θ (the angle subtended by the positively charged residues) from 100° to 140° thereby delocalizing the positive charge over a greater surface area of the molecule. In the case of the G4K and N8K analogs, the lysine residues are concentrated on polar patch on the hydrophilic face of the helix (θ = 100°) and this increase in amphipathicity is probably responsible for the increased cytotoxicity against human erythrocytes (*22*).

Table 1. Naturally occurring antimicrobial peptides from frog skin with potential for development into potent, nontoxic, anti-infective agents for use against antibiotic-resistant bacteria

Frog species	*Naturally occurring antimicrobial peptide*	*Primary structure*	*Microbial Selectivity*
Midwife toad *Alytes obstetricans*	Alyteserin-1c	GLKDIFK-AGLGSLVK-GIAAHVAN[a]	Gram-negative
Tailed frog *Ascaphus truei*	Ascaphin-8	GFKDLLK-GAAKALVK-TVLF[a]	Broad-spectrum including *Candida* spp.
Foothill yellow-legged frog *Rana boylii*	Brevinin-1BYa	FLPILASLAAK-FGPKLF-CLVTKKC	Broad-spectrum including *Candida* spp.
Hokkaido frog *Rana pirica*	Brevinin-2PRa	GLMSLFKGVLK-TAGKHIFKNVG-GSLLDQAKCKIT-GEC	Broad spectrum
Mink frog *Lithobates septentrionalis*	Brevinin-2 related peptide (B2RP)	GI-WDTIKSMGKVFAGK-ILQNL[a]	Broad-spectrum including *Candida* spp.
Green paddy frog *Hylarana erythraea*	Brevinin-2-related peptide-ERa	GVIKSVLKGVAK-TVALGML[a]	Broad spectrumr
African running frog *Kassina senegalensis*	Kassinatuerin-1	GFMKYIG-PLIPHAVKAIS-DLI[a]	Broad spectrum
Paradoxical frog *Pseudis paradoxa*	Pseudin-2	GLNALKKVFQGI-HEAIKLINNHVQ	Gram-negative
California red-legged frog *Rana draytonii*	Temporin-DRa	HFLGTLVN-LAKKIL[a]	Gram-positive

[a] denotes C-terminal α-amidation

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Table 2. Transformation of naturally occurring cytotoxic frog skin antimicrobial peptides into nontoxic anti-infective agents with therapeutic potential by appropriate substitutions of amino acids

Naturally occurring peptide	*Nontoxic analog*	*Microorganisms targeted*
Alyteserin-1c	[E4K]alyteserin-1c	Multidrug-resistant *Acinetobacter baumannii*
Ascaphin-8	[L18K]ascaphin-8	Extended-spectrum β-lactamase (ESBL) *Klebsiella pneumoniae*
Brevinin-1BYa	[C18S,C24S]brevinin-1BYa	Azole-resistant *Candida* spp
B2-RP	[D4K,L18K]B2-RP	Multidrug-resistant *Acinetobacter baumannii*
Kassinatuerin-1	[G7k,S18k,D19k]kassinatuerin-1	Antibiotic-resistant *Escherichia coli*
Pseudin-2	[N3k,Q10k,E14k]pseudin-2	Antibiotic-resistant *Escherichia coli*
Temporin-DRa	[V7K]temporin-DRa	Methicillin-resistant *Staphylococcus aureus*
XT-7	[G4K]XT-7	Methicillin-resistant *Staphylococcus aureus*

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Brevinin-2-Related Peptide

Brevinin-2-related peptide (B2RP) was first isolated from the North American the mink frog *Lithobates septentrionalis* (*23*) and a structurally similar peptide, B2RP-ERa was isolated from skin secretions of the South-East Asian Green Paddy frog *Hylarana erythraea* (formerly *Rana erythraea*) (*24*).

These C-terminally α-amidated peptides show limited structural similarity to brevinin-2 peptides isolated from other Asian species but lack the C-terminal cyclic heptapeptide domain (Cys-Lys-Xaa$_4$-Cys). They represent peptides with therapeutic potential for treatment of infections produced by multidrug-resistant strains of *Acinetobacter baumannii* (MDRAB).

There has been a dramatic increase in the number of hospital acquired infections caused by the opportunistic Gram-negative pathogen *A. baumannii* during the past decade (*25*). These are typically encountered in immunocompromised and critically ill patients in intensive-care and burns units. However, reports of increasing incidence of community-acquired infections (*26*) and infections of military personnel with war wounds (*27*) mean that *A. baumannii* represents a serious threat to public health. Among strains causing nosocomial outbreaks, resistance to fluoroquinolones, aminoglycosides, sulphonamides, third-generation cephalosporins and even carbapenems are common. Treatments with alternative drugs such as polymyxins, particularly colistin (polymyxin E), and the glycylcycline, tigecycline are far from optimal due to concerns with nephrotoxicity regarding colistin and the bacteriostatic nature of tigecycline (*28*). Furthermore, increasing use of these antibiotics is already leading to the emergence of resistant strains. The antibiotic resistance of *A. baumannii* arises from a combination of different possible mechanisms: production of hydrolysing enzymes, activation of multi-drug efflux pumps, modification of the drug target, and poor penetration due to loss of porins (*29*). These mechanisms are unlikely to reduce the efficacy of antimicrobial peptides.

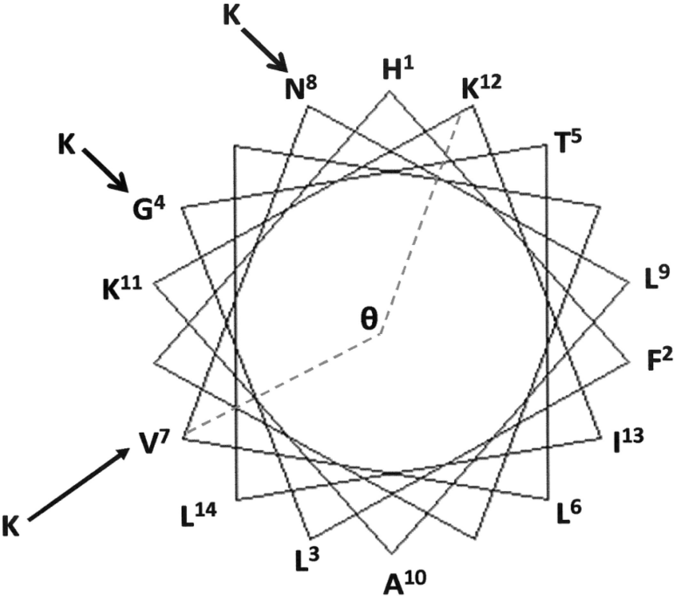


Figure 1. A Schiffer-Edmundson helical wheel projection of temporin-DRa illustrating the amphipathic nature of the α-helical conformation. Substitution of Val^7 *by L-Lys produces an analog with reduced hemolytic activity whereas substitution of Gly*^{4} *and Asn*^{8} *by L-Lys produces analogs with increased hemolytic activity.*

B2RP potently inhibited the growth of nosocomial isolates of multidrug-resistant *A. baumannii* (MIC = 3 - 6 μM). B2RP also shows relatively high potency (MIC ≤ 25 μM) against Gram-positive and Gram-negative bacteria and against the opportunistic yeast pathogen *C. albicans* but its therapeutic potential is limited by moderate hemolytic activity against human erythrocytes (LC_{50} = 90 μM) (*30*). Increasing cationicity of B2RP without changing amphipathicity by the substitution Asp^4→Lys resulted in increased potency against MDRAB isolates (MIC = 1.5 - 3 μM) and a 4-fold increase in potency against *E. coli* (MIC = 6 μM) and 2-fold increases in potency against *S. aureus* (MIC = 12.5 μM) and *Candida albicans* (MIC = 6 μM) without changing significantly hemolytic activity against human erythrocytes (LC_{50} = 95 μM). The analogs [D4K, L18K]B2RP

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

and [D4K,A16K, L18K]B2RP showed reduced potency against *S. aureus* but they retained activity against *A. baumannii* (MIC = 3 - 6 μM) and had very low hemolytic activity (LC_{50} > 400 μM).

B2RP-ERa showed particularly high potency against multi-drug resistant strains of *Stenotrophomonas maltophilia* (MIC in the range 2 - 4 μM; Minimum Bactericidal Concentration, MBC in the range 4 - 8 μM) and time-kill assays demonstrated that the peptide at a concentration of 2 x MBC produced 99.9% cell death within 30 min. B2RP-ERa was also active against clinical strains of MRSA belonging to different epidemic clonal lineages with MIC values in the range 25 to 50 μM. In time-kill kinetic assays, B2RP at a concentration of 2 x MIC was bacteriostatic but at a concentration of 4 × MIC the peptide was bactericidal with 99.9% of bacteria killed within 24 hours. The hemolytic activity of the peptide was relatively low (LC_{50} = 280 μM) (N. Al-Ghaferi and J.M. Conlon, unpublished data).

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Alyteserin-1c

Alyteserin-1c, isolated from skin secretions of the midwife toad *Alytes obstetricans* (*31*) displays potent activity against clinical isolates of MDRAB (MIC = 5 - 10 μM; MBC = 5 - 10 μM) while displaying low hemolytic activity against human erythrocytes (LD_{50} = 220 μM) (*31*). Increasing the cationicity of alyteserin-1c by the substitution Glu^4 → Lys enhanced the potency against MDRAB (MIC = 1.25 - 5 μM; MBC = 1.25 - 5 μM) as well as decreasing hemolytic activity (HC_{50} > 400 μM). The bactericidal action of the analog was rapid with more than 99.9% of the bacteria being killed within 30 min at a concentration of 1 x MBC. Increasing the cationicity of [E4K]alyteserin-1c further by the additional substitutions of Ala^8,Val^{14}, or Ala^{18} by L-Lys did not enhance antimicrobial potency. In an attempt to prepare a long-acting analog of alyteserin-1c suitable for systemic use, a derivative of [E4K]alyteserin-1c containing a palmitate group coupled to the α-amino group at the N-terminus was synthesized. The peptide retained antimicrobial activity against MDRAB but showed dramatically increased hemolytic activity (> 40-fold) (*32*).

Ascaphin-8

Ascaphin-8 is a cationic α-helical peptide isolated from skin secretions of the tailed frog *Ascaphus truei* that shows broad-spectrum antibacterial activity but is also moderately toxic to human erythrocytes (LC_{50} = 55μM) (*33*). The peptide shows potential for treatment of infections produced by extended-spectrum β-lactamase-producing microorganisms.

Bacteria which possess extended-spectrum β-lactamases (ESBLs) have the capacity to hydrolyse a broad spectrum of beta-lactam antibiotics, including third generation cephalosporins (*34*). Originally observed in *Escherichia coli* and *Klebsiella spp.*, ESBL production has now been documented in other Gram-negative bacilli including *Proteus mirabilis*, *Citrobacter freundii, Shigella sonnei, Serratia marcescens, Acinetobacter* spp. and *Salmonella* spp. (*35*).

The epidemiology of ESBL producing Enterobacteriaceae is changing with the incidence of community-acquired infections progressively increasing (*36*). Treatment of patients with bacterial infections caused by such multi-resistant bacteria is challenging as antibiotic options are becoming increasingly limited.

All ESBL-producing clinical isolates of *Klebsiella pneumoniae* (MIC = 12.5 – 25 μM) and *Escherichia coli* (MIC = 1.5 - 6 μM) tested were susceptible to ascaphin-8, as well as a group of miscellaneous ESBL-producing strains (*Citrobacter*, *Salmonella*, *Serratia*, *Shigella* spp.) (MIC ≤ 25μM) (*37*). Analogs of ascaphin-8 in in which the amino acids at positions 10, 14, or 18 were replaced by lysine retained potent antibacterial activity while showing very low hemolytic activity (LC_{50} > 500 μM). Unexpectedly, ESBL-producing strains of *Proteus mirabilis* were susceptible to ascaphin-8 (MIC = 12.5 - 25 μM) although non-ESBL isolates of this organism were resistant to these peptides (MIC > 100 μM).

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Pseudin-2

Pseudin-2, a 24 amino-acid-residue antimicrobial peptide first isolated from the skin of the South American paradoxical frog *Pseudis paradoxa* (*38*), also shows potential for treatment of infections caused by ESBL-producing Gram-negative bacteria, particularly *E. coli*. The naturally occurring peptide has weak hemolytic activity but also relatively low potency against microorganisms. However, analogs of the peptide with increased cationicity and decreased α-helicity showed improved therapeutic properties (*39*). [D-Lys^3, D-Lys^{10}, D-Lys^{14}]pseudin-2 showed potent activity against Gram-negative bacteria (MIC against several antibiotic-resistant strains of *E. coli* = 5 μM) but very low hemolytic activity (HC_{50} > 500 μM) and cytolytic activity against L929 fibroblasts (LC_{50} = 215 μM). Time-kill studies demonstrated that the analog at a concentration of 1 x MIC was bactericidal against *E. coli* (99.9% cell death after 96 min) but was bacteriostatic against *S. aureus*.

Kassinatuerin-1

Kassinatuerin-1, a 21-amino-acid C-terminally α-amidated peptide isolated from the skin of the African frog *Kassina senegalensis*, shows broad-spectrum antimicrobial activity but its therapeutic potential is limited by its relatively high cytolytic activity against mammalian cells (*40*). Analogs containing L-lysine substitutions at Gly^7, Ser^{18}, and Asp^{19} displayed increased antimicrobial potency but also increased hemolytic activities. In contrast, the analog with D-lysine at positions 7, 18 and 19 was active against a range of strongly antibiotic-resistant strains of *E. coli* (MIC = 6 - 12.5 μM) but showed no detectable hemolytic activity at 400 μM. However, the reduction in α-helicity produced by the D-amino acid substitutions resulted in analogs with reduced potencies against Gram-positive bacteria and against the opportunistic yeast pathogen *C. albicans* (*41*).

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Brevinin-1BYa

Brevinin-1BYa is a cationic α-helical peptide containing an intramolecular disulphide bridge that was first isolated from skin secretions of the foothill yellow-legged frog *Rana boylii* (*42*). The peptide shows potential for treatment of infection caused by azole-resistant *Candida* spp. The widespread use of azoles has led to the rapid development of multidrug resistance in *C. albicans* and other *Candida* species, which poses a major problem for antifungal therapy (*43*). Patients in ICU, undergoing abdominal surgery (*44*), or prolonged immunosuppressive therapy for transplants or treatment of malignancy (*45*), and patients with indwelling devices (*46*) are particularly at risk for nosocomial *Candida* infections.

As well as showing growth inhibitory activity against a range of reference strains of Gram-positive and Gram-negative bacteria and against clinical isolates of MRSA (MIC = 2.5 μM), the peptide was active against reference strains and clinical isolates of the opportunistic yeast pathogens *C. albicans*, *C. tropicalis*, *C krusei* and *C. parapsilosis* (MIC ≤ 10 μM) (*47*). However, the therapeutic potential of the peptide, especially for systemic applications, is restricted by its high hemolytic activity against human erythrocytes (LD_{50} = 10 μM). Replacement of the cysteine residues in brevinin-1BYa by serine produced an acyclic analogue with eight-fold reduced hemolytic activity that retained high potency against strains of MRSA (MIC = 5 μM) but activities against yeast species were reduced (MIC in the range 10 - 40 μM). More recently, a cyclic analog of brevinin-1BYa was prepared in which the intramolecular disulphide bridge in the peptide was replaced by a metabolically stable, non-reducible dicarba bond. The resulting compound showed increased antifungal activity (MIC against *C. albicans* = 3 μM) but this advantage was offset by increased hemolytic activity (LD_{50} = 4 μM) (*48*).

Brevinin-2PRa

Brevinin-2PRa was isolated from an extract of the skin of the Hokkaido frog, *Rana pirica* (*49*) and is a candidate for development into an anti-infective agent for use against antibiotic-resistant *Pseudomonas aeruginosa*. This opportunistic Gram-negative bacillus is characterized by its intrinsic resistance to several antibiotics and for its abilities to colonize diverse habitats and cause serious disease in vulnerable populations (*50*). The bacterium is found in low concentrations amongst the intestinal and skin flora of healthy humans but in compromised hosts, such as immunosuppressed patients and those with neutropenia, burns, cancer, diabetes mellitus, and chronic lung disease, it is responsible for life-threatening infections (*51*). In particular, *P. aeruginosa* is the major pathogen in the lungs of patients with cystic fibrosis where its survival is enhanced by conversion to biofilm-growing mucoid (alginate-producing) strains (*52*). Hospitals represent a reservoir of drug-resistant strains so that nosocomial infections of the respiratory and urinary tracts constitute a growing problem (*53*).

Brevinin-2PRa displayed high potency (MIC values between 6 and 12 μM) against a range of clinical isolates of *P. aeruginosa* with varying degrees of antibiotic resistance and activity was unaffected by NaCl concentrations up to 200 mM (*49*). The peptide was also active against reference strains of other Gram-negative (*E. coli*, *Enterobacter cloacae*, and *K. pneumoniae*) and Gram positive (*S. aureus, S. epidermidis*) bacteria but displayed moderate hemolytic activity (LC = 55 μM).

Future Clinical Applications of Frog Skin Antimicrobial Peptides

For progress in the field to continue, new clinical applications for frog skin antimicrobial peptides need to be found. *Acne vulgaris* is a disease of the pilosebaceous unit with both bacterial and inflammatory components. The Gram-positive anaerobic bacillus *Propionibacterium acnes* is found in normal human cutaneous flora and colonisation and proliferation by this organism play a major role in the development of an acne lesion (*54*). Bacterial colonisation is preceded by hyperproliferation of keratinocytes and increased sebum secretion in a hair follicle together with stimulation of release of proinflammatory cytokines and prostaglandins by follicular keratinocytes, mononuclear cells, and macrophages (*55*). Antibiotic resistance in *P. acnes* following prolonged monotherapy has been documented (*56*).

Several frog skin peptides have shown potent growth-inhibitory activity (MIC < 10 μM) against isolates of *P. acnes* from blood cultures. These include the naturally occurring acyclic brevinin-1-related peptide RV-23 (originally described as a melittin-related peptide) from *R. draytonii* (*57*), [T5k]temporin-DRa, [GK4]XT-7, and B2RP (E. Urbán and J.M. Conlon, unpublished data). Previous studies have shown that cationic antimicrobial peptides, as well as possessing microbicidal actions, will inhibit the release of proinflammatory cytokines and so may reduce the inflammatory response that follows bacterial skin colonisation (*58*, *59*). B2RP and [G4K]XT-7 inhibit release of proinflammatory tumor necrosis factor-α from human mononuclear cells and stimulate release of the anti-inflammatory cytokines interleukin-4 and interleukin-10 (S. Popovic, M. L. Lukic and J.M. Conlon, unpublished data). The peptides may thus exercise a dual beneficial role in acne treatment by manifesting a bactericidal action on *P. acnes* and an anti-inflammatory effect on host cells.

In a similar manner, the formation of microbial biofilms in the oral cavity can initiate a cascade of inflammatory responses that lead to the destruction of gingival tissues and ultimately tooth loss. There is an extensive literature relating to antimicrobial peptides and proteins in saliva and gingival crevicular fluid that provide protection against pathogenic microorganisms (*60*). Magainin and selected analogs show potent and rapid bactericidal activity against a range of anaerobic oral pathogens such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella* spp. (*61*). More recently, caerulein precursor fragment CPF-AM1 from the African clawed frog *Xenopus amieti* (*62*) has shown particularly high potency (MIC < 2.5 μM) against the cariogenic microorganisms *Streptococcus mutans*, and *Lactobacillus acidophilus* (F. Lundy and J.M. Conlon,

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

unpublished data). Consequently, a role of frog skin peptides in the prevention and treatment of periodontal disease is a possibility.

Conclusion

Studies with frog skin antimicrobial peptides indicate that small changes in structure can dramatically alter the relative cytolytic activities against bacterial and mammalian cells. Structure-activity investigation with a range of peptides suggest a strategy of selective increases in cationicity concomitant with decreases in helicity, hydrophobicity, and amphipathicity (increase in polar angle) in the transformation of naturally-occurring antimicrobial peptides into nontoxic agents with therapeutic potential for use against microorganisms that have developed resistance to currently licensed antibiotics.

Acknowledgments

The experimental work carried out at U.A.E. University was supported by Faculty Support Grants and Interdisciplinary Grants from the institution and by the Terry Fox Fund for Cancer Research.

References

1. Norrby, S. R.; Nord, C. E.; Finch, R. *Lancet Infect. Dis.* **2005**, *5*, 115–119.
2. Livermore, D. M. *J. Antimicrob. Chemother.* **2009**, *64* (Suppl 1), i29–36.
3. Diamond, G.; Beckloff, N.; Weinberg, A.; Kisich, K. O. *Curr. Pharm. Des.* **2009**, *15*, 2377–2392.
4. Zaiou, M. *J. Mol. Med.* **2007**, *85*, 317–329.
5. Perron, G. G.; Zasloff, M.; Bell, G. *Proc. Biol. Sci.* **2006**, *273*, 251–256.
6. Zasloff, M. *N. Engl. J. Med.* **2002**, *347*, 1199–1200.
7. Rinaldi, A. C. *Curr. Opin. Chem. Biol.* **2002**, *6*, 799–804.
8. Conlon, J. M. *Cell Tissue Res.* **2011**, *343*, 201–212.
9. Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449–5453.
10. Giovannini, M. G.; Poulter, L.; Gibson, B. W.; Williams, D. H. *Biochem. J.* **1987**, *243*, 113–120.
11. Abbassi, F.; Lequin, O.; Presse, C.; Goasdoué, N.; Foulon, T.; Nicolas, P.; et al. *J. Biol. Chem.* **2010**, *285*, 16880–16892.
12. Powers, J. P; Hancock, R. E. *Peptides* **2003**, *24*, 1681–1691.
13. Yeaman, M. R.; Yount, N. Y. *Pharmacol. Rev.* **2003**, *55*, 27–55.
14. Conlon, J. M.; Kolodziejek, J.; Nowotny, N. *Biochim. Biophys. Acta* **2009**, *1788*, 1556–1563.
15. Tennessen, J. A.; Woodhams, D. C.; Chaurand, P.; Reinert, L. K.; Billheimer, D.; Shyr, Y.; et al. *Dev. Comp. Immunol.* **2009**, *33*, 1247–1257.
16. Conlon, J. M.; Al-Ghaferi, N.; Abraham, B.; Leprince, J. *Methods* **2007**, *42*, 349–357.
17. Ge, Y.; MacDonald, D. L.; Holroyd, K. J.; Thornsberry, C.; Wexler, H.; Zasloff, M. *Antimicrob. Agents Chemother.* **1999**, *43*, 782–788.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

18. Conlon, J. M.; Al-Ghaferi, N.; Coquet, L.; Leprince, J.; Jouenne, T.; Vaudry, H.; et al. *Peptides* **2006**, *27*, 1305–1312.
19. Lowy, F. D. *N. Engl. J. Med.* **1998**, *339*, 520–532.
20. Zetola, N.; Francis, J. S.; Nuermberger, E. L.; Bishai, W. R. *Lancet Infect. Dis.* **2005**, *5*, 275–286.
21. Woodford, N.; Livermore, D. M. *J. Infect.* **2009**, *59* (Suppl. 1), S4–S16.
22. Dathe, M.; Wieprecht, T.; Nikolenko, H.; Handel, L.; Maloy, W. L.; MacDonald, D. L.; et al. *FEBS Lett.* **1997**, *403*, 208–212.
23. Bevier, C. R.; Sonnevend, A.; Kolodziejek, J.; Nowotny, N.; Nielsen, P. F.; Conlon, J. M. *Comp. Biochem. Physiol. C* **2004**, *139*, 31–38.
24. Al-Ghaferi, N.; Kolodziejek, J.; Nowotny, N.; Coquet, L.; Jouenne, T.; Leprince, J.; et al. *Peptides* **2010**, *31*, 548–554.
25. Neonakis, I. K.; Spandidos, D. A.; Petinaki, E. *Int. J. Antimicrob. Agents* **2011**, *37*, 102–109.
26. Karageorgopoulos, D. E.; Falagas, M. E. *Lancet Infect. Dis.* **2008**, *8*, 751–762.
27. Sebeny, P. J.; Riddle, M. S.; Petersen, K. *Clin. Infect. Dis.* **2008**, *47*, 444–449.
28. Giamarellou, H.; Poulakou, G. *Drugs* **2009**, *69*, 1879–1901.
29. Fishbain, J.; Peleg, A. Y. *Clin. Infect. Dis.* **2010**, *51*, 79–84.
30. Conlon, J. M.; Ahmed, E.; Condamine, E. *Chem. Biol. Drug Des.* **2009**, *74*, 488–393.
31. Conlon, J. M.; Demandt, A.; Nielsen, P. F.; Leprince, J.; Vaudry, H.; Woodhams, D. C. *Peptides* **2009**, *30*, 1069–1073.
32. Conlon, J. M.; Ahmed, E.; Pal, T.; Sonnevend, A. *Peptides* **2010**, *31*, 1806–1810.
33. Conlon, J. M.; Sonnevend, A.; Davidson, C.; Smith, D. D.; Nielsen, P. F. *Biochem. Biophys. Res. Commun.* **2004**, *320*, 170–175.
34. Paterson, D. L.; Bonomo, R. A. *Clin. Microbiol. Rev.* **2005**, *18*, 657–686.
35. Pitout, J. D. *Drugs* **2010**, *70*, 313–333.
36. Livermore, D. M.; Canton, R.; Gniadkowski, M.; Nordmann, P.; Rossolini, G. M.; Arlet, G.; et al. *J. Antimicrob. Chemother.* **2007**, *59*, 165–174.
37. Eley, A.; Ibrahim, M.; Kurdi, S. E.; Conlon, J. M. *Peptides* **2008**, *29*, 25–30.
38. Olson, L.; Soto, A.; Knoop, F. C.; Conlon, J. M. *Biochem. Biophys. Res. Commun.* **2001**, *288*, 1001–1015.
39. Pál, T.; Sonnevend, A.; Galadari, S.; Conlon, J. M. *Regul. Pept.* **2005**, *129*, 85–91.
40. Matutte, B.; Knoop, F. C.; Conlon, J. M. *Biochem. Biophys. Res. Commun.* **2000**, *268*, 33–36.
41. Conlon, J. M.; Abraham, B.; Galadari, S.; Knoop, F. C.; Sonnevend, A.; Pál, T. *Peptides* **2005**, *26*, 2104–2110.
42. Conlon, J. M.; Sonnevend, A.; Patel, M.; Davidson, C.; Nielsen, P. F.; Pál, T. *J. Pept. Res.* **2003**, *62*, 207–213.
43. Miceli, M. H.; Díaz, J. A.; Lee, S. A. *Lancet Infect. Dis.* **2011**, *11*, 142–151.
44. Arendrup, M. C. *Curr. Opin. Crit. Care* **2010**, *16*, 445–452.
45. Richardson, M. D. *J. Antimicrob. Chemother.* **2005**, *56* (Suppl. 1), i5–i11.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch003

46. Pierce, G. E. *J. Ind. Microbiol. Biotechnol.* **2005**, *32*, 309–318.
47. Pál, T.; Abraham, B.; Sonnevend, A.; Jumaa, P.; Conlon, J. M. *Int. J. Antimicrob. Agents* **2006**, *27*, 525–529.
48. Hossain, M. A; Guilhaudis, L.; Sonnevend, A.; Attoub, S.; van Lierop, B. J.; Robinson, A. J.; et al. *Eur. Biophys. J.* **2011**, *40*, 555–564.
49. Conlon, J. M.; Sonnevend, A.; Patel, M.; Al-Dhaheri, K.; Nielsen, P. F.; Kolodziejek, J.; et al. *Regul. Pept.* **2004**, *118*, 135–141.
50. Strateva, T.; Yordanov, D. *J. Med. Microbiol.* **2009**, *58*, 1133–1148.
51. Driscoll, J. A.; Brody, S. L.; Kollef, M. H. *Drugs* **2007**, *67*, 351–368.
52. Høiby, N.; Ciofu, O.; Bjarnsholt, T. *Future Microbiol.* **2010**, *5*, 1663–1674.
53. Mesaros, N.; Nordmann, P.; Plésiat, P.; Roussel-Delvallez, M.; Van Eldere, J.; Glupczynski, Y.; et al. *Clin. Microbiol. Infect.* **2007**, *13*, 560–578.
54. Bhambri, S.; Del Rosso, J. Q.; Bhambri, A. J. *Drugs Dermatol.* **2009**, *8*, 615–618.
55. Kurokawa, I.; Danby, F. W.; Ju, Q.; Wang, X.; Xiang, L. F.; Xia, L.; et al. *Exp. Dermatol.* **2009**, *18*, 821–832.
56. Patel, M.; Bowe, W. P.; Heughebaert, C.; Shalita, A. R. *J. Drugs Dermatol.* **2010**, *9*, 655–664.
57. Urbán, E.; Nagy, E.; Pál, T.; Sonnevend, A.; Conlon, J. M. *Int. J. Antimicrob. Agents* **2007**, *29*, 317–321.
58. Marta Guarna, M.; Coulson, R.; Rubinchik, E. *FEMS Microbiol. Lett.* **2006**, *257*, 1–6.
59. Bowdish, D. M.; Davidson, D. J.; Scott, M. G.; Hancock, R. E. *Antimicrob. Agents Chemother.* **2005**, *49*, 1727–1732.
60. Gorr, S. U.; Abdolhosseini, M. *J. Clin. Periodontol.* **2011**, *38* (Suppl. 11), 126–141.
61. Genco, C. A.; Maloy, W. L.; Kari, U. P.; Motley, M. *Int. J. Antimicrob. Agents* **2003**, *21*, 75–78.
62. Conlon, J. M.; Al-Ghaferi, N.; Ahmed, E.; Meetani, M. A.; Leprince, J.; Nielsen, P. F. *Peptides* **2010**, *31*, 989–994.

Chapter 4

Obstacles and Solutions to the Use of Cationic Antimicrobial Peptides in the Treatment of Cancer

Ashley L. Hilchie,[1,§] Melanie R. Power Coombs,[2,§]
and David W. Hoskin*,[1,2,3]

[1]Department of Microbiology & Immunology, Faculty of Medicine,
Dalhousie University, Halifax, Nova Scotia, Canada
[2]Department of Pathology, Faculty of Medicine, Dalhousie University,
Halifax, Nova Scotia, Canada
[3]Department of Surgery, Faculty of Medicine, Dalhousie University,
Halifax, Nova Scotia, Canada
*E-mail: D.W.Hoskin@dal.ca
§Equal contributers to this work.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

Cationic antimicrobial peptides (CAPs) are cytotoxic agents that show promise for use in conjunction with current anti-cancer therapies to improve the specific killing of cancer cells. CAPs are small peptides that constitute an important innate defense mechanism against microbial pathogens in many different species. CAPs that are selectively cytotoxic for cancer cells, regardless of their growth rate or expression of multidrug-resistance proteins, show considerable promise as an alternative to conventional chemotherapy. Murine models of cancer indicate that many CAPs can target and kill cancer cells without causing undue harm to normal tissues. In order for an anti-cancer CAP to be clinically useful, the CAP must be specific for cancer cells, stable in serum, cost effective, and minimally immunogenic. Herein, obstacles and possible strategies to using CAPs in the treatment of cancer are discussed. Thus far, preclinical studies provide a strong rationale for the possible clinical use of CAPs in cancer patients.

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Introduction

Cationic antimicrobial peptides (CAPs) are small peptides, usually fewer than 40 amino acids (aa) in length, that are predominantly composed of positively charged (e.g., lysine and arginine) and hydrophobic (e.g., tryptophan) aa (*1*, *2*). CAPs typically adopt amphipathic secondary structures when they come in contact with biological membranes. The amphipathic secondary structure typically consists of a predominantly cationic face, which is thought to initiate CAP binding to negatively charged structures on the cell surface, and a hydrophobic face, which is believed to be mediate membrane destabilization (*2–5*). CAPs are typically classified as α-helical, β-sheet, loop or extended peptides based on the secondary structure that they adopt upon contact with biological membranes (*6*). Certain CAPs kill Gram-negative and Gram-positive bacteria (*7–12*), viruses (*10*, *13*), fungi (*14*), parasites (*15–19*) and cancer cells (*20–29*). These properties make CAPs a vital component of the innate immune system of many organisms, including insects, fish, and mammals, and suggest possible clinical application in the treatment of human diseases such as cancer.

Both direct and indirect mechanisms are responsible for CAP-mediated killing of cancer cells. Direct-acting CAPs kill cancer cell cells by causing significant and irreversible membrane damage that leads to cell lysis, whereas indirect-acting CAPs kill cancer cells by inducing apoptosis, inhibiting the synthesis of essential macromolecules, or triggering receptor-mediated alterations in signal transduction pathways (*30*). Interestingly, the concentration of the peptide can influence its mechanism of action. In this regard, lower concentrations of certain peptides kill bacteria by an indirect mechanism that involves inhibition of macromolecule synthesis, while higher concentrations of the same peptide kill by cytolysis (*9*). Similar findings have recently been reported in cancer cells (*25*). The capacity of certain peptides to kill cancer cells by multiple mechanisms gives CAPs an additional advantage over conventional chemotherapeutic drugs currently used in cancer treatment.

Several models have been proposed to describe the mechanism by which direct-acting CAPs cause membrane disruption (*2*, *3*, *31–33*). These models include the "barrel stave" model, the "carpet" model, and the "toroidal pore" or "two state" model. Although these models differ in some subtle details, all membrane disruption is initiated by peptide binding to the outer membrane leaflet of the cancer cell membrane via electrostatic interactions between the positively charged aa side chains of the CAP and negatively charged cell-surface molecules present on the outer membrane leaflet of the cancer cell. CAP binding to the cancer cell leads to the formation of a stable amphipathic secondary structure that likely results in the exposure of hydrophobic aa, which then may insert into the lipid core of the membrane, thereby anchoring the CAP to the membrane. The barrel-stave model describes a process whereby peptide monomers aggregate and form transmembrane pores composed solely of peptides that resemble staves in a barrel (*31*, *34*). In the carpet model, CAP monomers cover the surface of the target cell like a carpet, which causes membrane thinning and the formation of transient pores (*35*, *36*). The toroidal pore model combines elements of the barrel stave and carpet models. In this regard, membrane lysis occurs once a threshold

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

concentration of CAP monomers bind to the target cell in a parallel manner (*37*, *38*). In contrast to the barrel stave model, the toroidal pore model predicts that both CAPs and lipid molecules line the torus-shaped pore.

Potential Clinical Use of CAPs

Compared to normal cells, which contain zwitterionic lipids and are therefore neutral in charge, the outer membrane leaflet of cancer cells carries a net negative charge due to a greater abundance of phosphatidylserine residues, O-glycosylated mucins, heparan sulfate proteoglycans (HSPs), and sialylated glycoproteins (*39–42*). As a result, certain CAPs with anti-cancer properties bind neoplastic cells with a 10-fold greater binding affinity than normal cells (*18*), rendering these CAPs selectively cytotoxic for cancer cells. Increased transmembrane potential, surface area, and membrane fluidity, all of which are associated with neoplastic cells, may also enhance the selectivity of CAPs for cancer cells (*9*). Conventional chemotherapy indiscriminately targets rapidly dividing cells, including many normal cells (*43*). Consequently, chemotherapy fails to kill slow-growing or dormant cancer cells (*44*). The therapeutic utility of chemotherapy is further reduced by the emergence of tumour cell variants that overexpress multidrug-resistance proteins that enable the cancer cell to exclude the cytotoxic drug before it can cause cell death (*45*). CAPs with anti-cancer properties have the potential to be superior to conventional chemotherapeutic agents because CAPs rapidly bind to and kill a wide range of human cancer cells, including multidrug-resistant cancer cells, without causing undue harm to vital organs (*23*, *43*, *46–55*). Slow-growing cancers are predicted to be susceptible to CAP-mediated cytotoxicity since the net charge of the cell rather than its proliferative capacity determines susceptibility. Moreover, many different cell-surface molecules contribute to the negative charge of cancer cell membranes. Therefore, cancer cell resistance to CAPs seems unlikely - in fact, cancer cell resistance to membranolytic CAPs has never been documented. Finally, certain CAPs enhance the cytotoxic action of chemotherapeutic drugs, suggesting that these peptides may function as chemosensitizing agents (*46*, *50*).

In order to be clinically useful, anti-cancer CAPs must be specific for cancer cells, stable, cost effective, and minimally immunogenic. Many research groups have addressed these issues by designing peptide mimics, or peptidomimetics, that possess improved anti-cancer properties. These purpose-engineered CAPs may ultimately prove superior to conventional chemotherapeutic agents for the treatment of cancer.

Peptide Modification: Strategies That Improve the Anticancer Properties of CAPs

Three major short-comings limit the potential therapeutic value of CAPs: unacceptable cytotoxicity for normal cells, peptide degradation by proteases, and cost of production. Although most CAPs preferentially kill negatively charged cancer cells, cytotoxic effects on normal eukaryotic cells may be an issue with

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

some peptides (*56*, *57*). Increasing the positive charge and/or hydrophobicity of the CAP, substituting histidine for lysine and/or arginine residues, or adding cancer cell-targeting moieties are approaches to improving the selectivity of CAPs for cancer cells. Protease-mediated degradation of peptides occurs rapidly and significantly reduces the anti-cancer activites of CAPs. Incorporating enantiomeric aa (i.e., D-aa rather than L-aa) into the peptide results in increased CAP stability. Finally, it is important to note that clinical grade CAPs are expensive to produce. Researchers are therefore working to reduce the cost of peptide production by engineering truncated CAPs with anti-cancer properties that are equivalent to the full-length CAP. These modification strategies are summarized in Table I, and will be discussed below with an emphasis on how such approaches may influence cancer cell killing and/or peptide stability.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

Table I. Examples of CAP modification strategies to enhance peptide stability and specificity for cancer cells

Strategy	*CAP Modified*	*Type of Cancer*	*Model*	*Reference*
Modifying charge	Bovine Lf derivative	Human mammary & lung carcinomas	*In vitro*	(*58*)
Lys to His substitution	Synthetic peptide ([D]-K_6L_9)	Human prostate cancer	*In vitro & in vivo*	(*59*)
Targeting motif addition	Tachyplesin	Human prostate cancer & mouse melanoma	*In vitro & in vivo*	(*55*)
Cyclization	LfcinB	Leukemia, lymphoma & fibrosarcoma	*In vitro & in vivo*	(*23*, *60*, *61*)
Truncation	Bovine Lf derivative	Human mammary & lung carcinomas	*In vitro*	(*58*)
D-amino acid incorporation	Synthetic peptide (K_6L_9)	Human prostate cancer	*In vitro & in vivo*	(*62*)

Modification of Positive Charge and/or Hydrophobicity

Direct CAP-mediated cancer cell death begins with the peptide binding to the membrane of the cancer cell. Peptide binding is mediated by electrostatic interactions between the positively charged side chains of the CAP and the negatively charged structures present on the outer membrane leaflet of the cancer cell (*31*). Therefore, replacing anionic or neutral aa with cationic aa may increase the attraction between the CAP and the cancer cell. However, care must be taken

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

when selecting aa for replacement not to eliminate or impair the function of hydrophobic aa residues that are responsible for membrane destabilization and, ultimately, cell death (*6*, *63*). Additionally, the presence of bulky aa may help to protect the peptide backbone from protease-mediated digestion by "hiding" cleavage sites from their respective proteases. Moreover, aa other than cationic or hydrophobic residues may also be essential for CAP-mediated killing of cancer cells. For example, proline and glycine residues are helix-breaking aa that can be important for peptide-mediated anti-microbial and hemolytic activities (*64*, *65*). Furthermore, the secondary structure of the peptide can be compromised when the aa sequence is altered, which may lead to a loss of cytotoxic activity. Although several studies have investigated the value of manipulating charge and hydrophobicity on anti-microbial activity by CAPs (*66*, *67*), the impact of such modifications on their anti-cancer properties has not been well studied with few notable exceptions, such as bovine lactoferricin (LfcinB).

Lfcin is a 25-aa CAP that is released following pepsin cleavage of the NH_2 (N)-terminus of lactoferrin (Lf), a protein found in many mucosal secretions (*68*). LfcinB has greater anti-bacterial activity than human, murine, or caprine Lfcin (*69*). Linear and cyclic LfcinB, composed of aa corresponding to positions 17-41 of Lf, demonstrate anti-cancer activities *in vitro* and *in vivo* (*23*, *60*, *61*). Yang *et al.* evaluated the influence of manipulating the positive charge and hydrophobicity of peptide analogs derived from the N-terminal helical region of bovine Lf (residues 14-31) (*70*). In a helical wheel diagram of the native peptide, the cationic residues are clustered in two spatially separated sections called the minor and major sectors, which consist of two and four cationic aa, respectively. When the cationic residues of the minor sector are moved to the major sector the anti-cancer activity of the CAP increases. However, unlike the native peptide, the Lf derivative is cytotoxic to normal fibroblasts and red blood cells, suggesting that a minor sector may confer selectivity for cancer cells. Interestingly, increasing the charge from +6 to +8 does not significantly increase the peptide killing of normal cells, providing that two cationic aa remain in the minor sector (*70*). However, this modification results in reduced cancer cell killing by the CAP, presumably due to the loss of two bulky tryptophan residues since these aa are important for CAP-mediated membrane disruption (*63*). This conclusion was supported by a study by Strom *et al.* who demonstrated that the anti-bacterial activity of a 15-aa derivative of LfcinB is abolished when either tryptophan residue is replaced with an alanine residue (*71*).

Results from other studies that evaluated the influence of charge on the anti-cancer activity of Lf derivatives suggest that a charge of at least +7 and an amphipathic structure are required for cancer cell killing (*58*). However, the significance of this finding is diminished by reports that increasing the positive charge of a CAP may not translate to improved anti-cancer activity (*48*). These contradictory results may be explained by differences in peptide structure dictated by the primary aa sequence. Together, these findings suggest that the requirements for cancer cell killing may vary significantly between peptides, which supports the hypothesis that a delicate balance of charge and hydrophobicity are required to obtain selective CAP-mediated killing of cancer cells.

Although Yang *et al.* successfully enhanced peptide selectivity for cancer cells by modifying the aa sequence of analogs of bovine Lf's N-terminal helical

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

region (*58*), considerable time and resources were likely required to generate derivatives that were sufficiently selective for cancer cells. Therefore, application of this modification technique may be costly. Furthermore, if the CAP mediates cancer cell killing by an indirect receptor-dependent mechanism, changing the aa sequence at the receptor binding site would likely reduce the ability of the peptide to kill cancer cells. In the absence of adequate structural information, such as that obtained from nuclear magnetic resonance (NMR) studies, slight changes in the aa sequence could also lead to a secondary structure that is significantly different from that of the native CAP, which could result in reduced cytotoxicity. Therefore, it is important to be aware of the mode of action of the CAP and its structure before CAP modification is attempted using this technique.

Because the tumour microenvironment is typically acidic (*72*), it is possible to enhance the specificity and therefore reduce the toxicity of CAPs by performing lysine to histidine substitutions (*58*). Such peptides become cationic in the acidic microenvironment of solid tumours due to histidine protonation at pH values lower than 7. For example, Makovitzki *et al.* modified [D]-K_6L_9 by replacing lysine with histidine residues to generate the modified CAPs, [D]-$K_3H_3L_9$ and [D]-H_6L_9, that became protonated and activated in acidic environments (*59*). Growth of human prostate carcinoma xenografts in mice were inhibited by intratumoural injections (9 injections, 1 every second day) of [D]-$K_3H_3L_9$ or [D]-H_6L_9 (1 mg/kg), starting 1 week after implantation. Systemic administration of [D]-$K_3H_3L_9$ or [D]-H_6L_9 (9 mg/kg), 1 week after cancer cell implantation, also inhibited growth of human prostate carcinoma xenografts in mice (9 injections, one every second day) (*59*). Importantly, [D]-H_6L_9 has pH-dependent activity whereas [D]-$K_3H_3L_9$ does not. Interestingly, administration of [D]-$K_3H_3L_9$ or [D]-H_6L_9 did not result in any acute systemic toxicity at doses of up to 30 and 20 mg/kg, respectively, whereas [D]-K_6L_9 proved toxic at concentrations above 8 mg/kg (*59*). Substitution of lysine for histidine residues may therefore decrease the nonspecific toxicity of certain peptides, allowing for their systemic administration as well as rendering the CAPs more selective for solid tumours.

Targeting Motif Addition

Cancer cell selectivity can be further enhanced by conjugating CAPs to small peptide moieties that recognize molecules that are over-expressed by cancer cells (*51–55*, *73*). Several tumour-targeting peptide sequences have been identified that show promise as reliable cancer cell delivery vehicles (*74*), although studies evaluating the ability of tumour-targeting peptides to deliver cytotoxic CAPs to cancer cells are still in their infancy. Nevertheless, a few preliminary investigations have yielded promising results.

Conjugating the cyclic CAP tachyplesin to the integrin homing domain arginine-glycine-aspartic acid (RGD) yielded a hybrid peptide that induces apoptosis in cancer cell lines and inhibits tumour growth in two *in vivo* models: the chicken chorioallantoic membrane and syngenic mouse models (*55*). This finding suggests that the RGD sequence may enhance tachyplesin-mediated cancer cell killing while decreasing the chances of potentially toxic side effects. Since many cancer cells express luteinizing hormone releasing hormone (LHRH) receptors (*75*), linking cytotoxic CAPs with LHRH was predicted to promote peptide targeting of cancer cells. Indeed, cytolytic CAPs that are conjugated to LHRH destroy human prostate and breast cancer cells grown as xenografts in immune-deficient mice, as well as eliminating distant metastases (*52*, *76*). These findings suggest that certain targeted CAPs may be useful in the treatment of metastatic cancer.

Methods To Enhance CAP Stability

Several strategies exist to increase peptide stability in serum, including end capping, cyclization, D-aa or unnatural aa incorporation, peptoid synthesis, lysine acylation/alkylation, and peptide retro-inversion. The value of these various modification techniques is dependent on the structure, mode of action, and the target of the peptide of interest. It is difficult to predict which modification strategy will produce the most stable bioactive product without information on the structural and mechanistic properties of the peptide. Furthermore, at this moment it is impossible to predict the long-term consequences of administering cytotoxic peptides modified by these techniques. For example, significant enhancement of peptide stability may result in unpredictable *in vivo* toxicities if the modified peptide is not exquisitely selective for cancer cells. This potential danger highlights the need for both *in vitro* and *in vivo* comparative studies of peptides modified by each of the different modification strategies.

End Capping

End capping is a technique whereby the N- or carboxylic acid (C)-terminal regions of the CAP are chemically modified by N-terminal acetylation and C-terminal amidation, respectively (*77*). N-terminal acetylation can be used to compensate for an inherent lack of hydrophobicity (*78*). Lockwood *et al.* found that conjugating fatty acid moieties to the N-terminus of the CAP SC4 causes a 30-fold increase in its anti-bacterial activity (*79*). Unfortunately, the increase in anti-bacterial activity occurs at the expense of CAP selectivity for bacteria, which was indicated by an increase in peptide-mediated hemolysis. However, the peptide dose that caused hemolysis was still 10- to 100-fold higher than that required for anti-bacterial activity. Interestingly, the fatty acid moiety on the acetylated derivative stabilized the α-helical structure of the peptide in bacterial membrane-mimicking conditions, which may account for the CAP's increased potency. Together, these findings suggest that the acetylated hydrophobic N-terminus of SC4 interacts with the hydrophobic core of the membrane, which stabilizes the secondary structure of the CAP. Once stabilized, the CAP proceeds to disrupt the target cell membrane. The positive influence of N-acetylation on anti-bacterial activity is supported by the findings of Zweytick *et al.*, who showed that acetylating a 10-aa human Lfcin derivative increases its anti-bacterial activity (*80*). Taken together, these findings indicate that, as with incorporation of hydrophobic aa to increase CAP hydrophobicity, N-acetylation can raise CAP

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

hydrophobicity to the threshold level needed to cause extensive damage to the cell membrane.

C-terminal amidation is another capping technique that is used to enhance the anti-bacterial activity of CAPs by increasing the positive charge at the C-terminus. For example, C-terminal amidation enhances SC4-mediated anti-bacterial activity, although SC4-NH_2 proved to be hemolytic when employed at higher concentrations (*81*). Other groups have also successfully used C-terminal amidation to enhance peptide-mediated anti-bacterial activity (*82*, *83*); however, in these studies, C-terminal amidation also resulted in stronger hemolytic activity. Therefore, additional "tweaking" of the aa sequence may be necessary to generate peptides with improved selectivity for cancer cells without eliciting undesirable hemolytic activity (*82*). It is important to note, however, that N-terminal amidation does not always result in CAP-mediated hemolysis (Hilchie and Hoskin, unpublished data) and, therefore, should be considered as an option for modifying certain peptides.

Importantly, end capping also reduces CAP susceptibility to protease-mediated degradation. For example, Svenson *et al.* showed that C-terminal amidation increased the resistance of very small CAPs to degradation by trypsin and was associated with a reduction in the minimal inhibitory concentration (MIC) for the three bacterial strains that were tested (*77*). Interestingly, the length of the C-terminal capping moiety has a significant influence on the resistance of CAPs to trypsin-mediated degradation. Thus, trypsin resistance is increased by more than an order of magnitude when the aromatic rings of the C-terminal 4-biphenyl-capping moiety were two carbon atoms away from its amide nitrogen as compared to analogues with one or three carbon atoms separating the 4-biphenyl-capping moiety from the nitrogen atom. Conversely, N-terminal acetylation increased trypsin-mediated degradation of CAPs and changes the preferred trypsin cleavage site to an adjacent arginine residue. However, it is important to note that the influence of N-terminal acetylation on peptide susceptibility to trypsin-mediated degradation was not evaluated in the absence of C-terminal amidation. The authors suggest that adding bulky caps to the N-terminus generates a trypsin docking site that is required for peptide degradation. N-terminal capping may therefore not protect larger CAPs from trypsin-mediated degradation. However, end capping may protect CAPs from digestion by aminopeptidase and/or carboxypeptidase, which are involved in alimentary digestion and could limit the bioavailability of CAPs delivered by the oral route (*84*). Therefore, in the right context, end capping may be a useful strategy for enhancing CAP stability.

It is important to note that the majority of experiments that have examinined the functional difference between capped and non-capped CAPs were performed using model membranes and bacteria rather than cancer cells. Importantly, CAP-mediated anti-bacterial activity does not always equate to CAP-mediated killing of cancer cells owing to the differences between bacterial and cancer cell membranes. A comprehensive investigation of the cytotoxicity of capped and non-capped anti-cancer peptides for both cancer cells and normal cells is therefore urgently needed to determine whether capping increases or decreases the selectivity of different CAPs for cancer cells.

Cyclization

Cyclic peptides are less sensitive to protease degradation than their linear counterparts, owing in part to their inherent lack of C- and N-terminal exopeptidase binding sites. An important study by Rozek *et al.* showed that cyclic indolicidin is more resistant than linear indolicidin to trypsin-mediated digestion *in vitro* (*85*), suggesting that cyclic CAPs may also exhibit enhanced stability *in vivo*. Indeed, many CAPs, including Lfcin, defensins, protegrin and tachyplesin are cyclic peptides (*86–88*). Preliminary studies evaluating the importance of cyclicity for anti-bacterial activity by CAPs have been performed with interesting but sometimes conflicting results. Whereas some studies show that linear and cyclic LfcinB have similar anti-bacterial activities (*68*, *89*), another study demonstrates that acyclic LfcinB has significantly less bactericidal activity than cyclic LfcinB (*69*). These conflicting findings may be explained by the differences in the bacterial strains that were used to test for LfcinB-mediated killing of bacteria. Yet another study demonstrated that the anti-microbial properties of an 11-aa LfcinB derivative are retained when the peptide is extended by two cysteine residues that form a disulphide bond; however, neither LfcinB derivative was cytotoxic for a neuroblastoma cell line, whereas full-length LfcinB was able to inhibit the growth of neuroblastoma cells (*86*). It remains to be established whether changing the cyclicity of a CAP impacts on its ability to kill cancer cells. Although one report indicates that cyclic LfcinB, but not linear LfcinB, possesses anti-cancer properties *in vivo* (*60*), other studies show that linear LfcinB has anti-cancer activities *in vivo* (*61*). Additional comparative studies of anti-cancer properties of a range of linear and cyclic CAPs therefore need to be conducted before the influence of peptide cyclization on cancer cell killing can be fully understood.

The anti-microbial activity of acyclic CAPs does not appear to be equivalent to that of the cyclic native peptide. A study by Tamamura *et al.* demonstrated that the loss of the two disulfide bonds in tachyplesin, a CAP isolated from the hemocyte extracts of the horseshoe crab, causes a decrease in its anti-bacterial activity (*90*). In addition, an investigation that used model membrane systems demonstrated that the mechanism of anti-bacterial activity of linear tachyplesin is different from that of cyclic tachyplesin, as evidenced by a reduction in calcein release and micellization in linear tachyplesin-treated model membranes compared to cyclic tachyplesin-treated model membranes (*91*). However the mechanistic difference between the two CAPs was not elucidated.

Interestingly, the therapeutic utility of certain CAPs can be enhanced by reduction to their linear form. Certain derivatives of acyclic human β-defensin 3 (HBD3) retain their anti-bacterial activity (*92*). In this study, one of six derivatives was hemolytic, and three of six derivatives were not cytotoxic for normal epithelial cells. These findings suggest that reducing the disulphide bond of certain CAPs may improve their selectivity for bacteria. Nevertheless, care must be taken when linearizing peptides because certain cyclic CAPs, including human β-defensins, have other important activities, such as immunomodulation (*93*). The consequence of removing or adding disulphide bonds on these other activities varies significantly between peptides and cannot be easily predicted.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

Truncation

The truncation or shortening of CAPs has several implications for peptide function and stability. Importantly, truncated CAPs may have decreased immunogenicity (*94*), which is expected to significantly improve their therapeutic potential. Short peptides are also predicted to be less susceptible to proteolytic degradation because trypsin prefers to act on substrates of a particular length (*95*, *96*). However, this prediction has been disputed by Svenson *et al.*, who demonstrated that trypsin is able to rapidly degrade small (<6 aa) peptides (*77*). Therefore, smaller peptides may require further modification to decrease their susceptibility to digestion by proteases. Perhaps the greatest benefit of developing truncated CAPs is that they are cheaper to produce. Therefore, many researchers are focusing on developing smaller CAPs that are as potent as full-length peptides, which may encourage their development as novel agents for the treatment cancer as well as other diseases.

The impact of truncation on CAP function has been evaluated in two bovine Lf derivatives, LfcinB and the N-terminal α-helical region of bovine Lf. Truncation of the N-terminal α-helical region of bovine Lf determined that a net charge close to +7 is essential for cytotoxic activity against cancer cells (*58*). However, the less cationic derivatives were also shorter than those with a net charge of +7 or more. In the event that these Lf derivatives adopt an α-helical structure, it is conceivable that the reduction in anti-cancer activity is caused by the inability of these derivatives to span the cancer cell membrane rather than by the lack of sufficient cationic charge. Interestingly, selected Lf derivatives that possess a charge less than +7 retain the ability to kill bacteria, supporting the notion that anti-bacterial activities do not necessarily predict anti-cancer activities of CAPs (*58*). Furthermore, the loss of amphipathicity in some Lf derivatives, based on predictions made by the Edmundson helical-wheel projection, is accompanied by diminished anti-cancer but not anti-bacterial activities. Taken together, these findings suggest that a higher positive charge and amphipathic secondary structure may be more important for direct killing of cancer cells than for anti-microbial activity.

The requirement for a minimum charge of +7 for CAP function (*58*) has been challenged by evidence that a 10-aa derivative of LfcinB with a charge of only +3 can cause cancer cell death (*23*). However, the 6-aa "anti-microbial core" of LfcinB which possesses a charge of +2 is not able to kill cancer cells unless it is transported into the cytosolic compartment (*97*). These conflicting findings can be explained by differences in the aa sequences between the two LfcinB derivatives, which dictates the secondary structure of the peptides. Together, these findings highlight the variability in the function of truncated CAPs. Nevertheless, a recent study found that the intratumoural injection of the CAP LTX-302, which is derived from LfcinB, caused tumour necrosis and infiltration of inflammatory cells followed by complete regression of B cell lymphoma tumours in the majority of immunocompetent mice (*98*). Mice that cleared the tumour after peptide injection were also protected against rechallenge and tumour resistance could be adoptively transferred with spleen cells from LTX-302-treated mice in a T cell-dependent manner. This important study suggests that the intratumoural administration of lytic peptides can generate local tumour control and may be useful as a therapeutic cancer vaccination strategy.

D-Amino Acid Incorporation

CAPs can be rendered resistant to proteolytic degradation by exchanging L-aa for D-aa, which are the chiral opposite of naturally-occurring L-aa (*99–102*). Several groups have evaluated the potential of D-aa incorporation to increase CAP stability by creating an enantiomer of the peptide under investigation by replacing all L-aa with D-aa, or by creating a diastereomeric isomer, in which those L-aa positioned at sites recognized by proteases are replaced with D-aa. Trypsin cleaves the peptide bond on the C-terminal side of arginine and lysine residues while chymotrypsin cleaves the peptide bond on the C-terminal side of the bulky hydrophobic residues phenylalanine, tyrosine, and tryptophan (*103*). Therefore, exchanging these residues in the CAP with their D-aa equivalent is predicted to significantly enhance CAP stability by decreasing it susceptibility to digestion by these common proteases. In this regard, all-D-pleurocidin, a CAP isolated from winter flounder, resists degradation by trypsin, plasmin and carboxypeptidase (*104*). Importantly, peptides composed of D-aa may be less immunogenic than peptides that contain L-aa because all-D-peptides cannot be processed by antigen presenting cells (*105*). Consistent with this prediction, BALB/C mice injected bi-weekly for five weeks with L-melittin showed robust production of anti-L-melittin IgG whereas mice treated with D-melittin produced only background amounts of anti-D-melittin IgG (*94*).

Several *in vitro* and *in vivo* studies have evaluated the influence of D-aa incorporation on CAP-mediated killing of cancer cells. Baker *et al.* compared the *in vivo* anti-cancer activities of magainin II and two magainin II derivatives: MSI-136, a modified form of magainin II, and MSI-238, an all-D-aa form of MSI-136 (*101*). An *in vitro* analysis revealed that MSI-238 is more potent than MSI-136, which is more potent than magainin II. Furthermore, a single treatment with MSI-238 significantly reduced the number and viability of P388D1 lymphoma cells in the ascites of tumour-bearing mice and significantly increased the life-span of mice with spontaneous ovarian teratomas. In addition, Papo *et al.* demonstrated that a synthetic diasterermeric CAP is resistant to trypsin-, elastase-, and proteinase-K-mediated degradation, and exhibits both *in vitro* and *in vivo* activity against cancer cells (*102*). Furthermore, intratumoural injection of a cytotoxic peptide composed of both D- and L-aa prevented the growth of prostate cancer xenografts and synergized with conventional chemotherapeutics (*62*). Taken together, these findings suggest that replacing select L-aa with D-aa may have the same effect on CAP stability *in vitro* and *in vivo* as complete replacement of L-aa with D-aa.

It is important to note that if the structure of a given CAP is the most important factor for anti-cancer activity, then the anti-cancer activity of its all-D-aa enantiomer should be equivalent to that of its all-L-aa form. However, if the CAP-mediated killing of cancer cells is a consequence of an interaction with a chiral center, such as a receptor, then the enantiomeric CAP will be less

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

potent than its naturally-occurring counterpart. Therefore, the mechanism by which a given CAP acts on target cells will largely determines the usefulness of this CAP modification strategy. For example, LfcinB inhibits the proliferation and migration of endothelial cells in response to the heparin-binding growth factors basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) by competing with growth factors for the same binding sites on cell-surface heparin sulfate proteoglycans (*106*). In contrast, an amino acid-scrambled form of LfcinB lacks inhibitory effects on endothelial cells. Thus, changing the chirality of LfcinB by incorporating D-aa would likely compromise the anti-angiogenic activities of LfcinB. For this reason, end capping may be a better strategy for increasing the stability of LfcinB, provided that the secondary structure of the resulting peptide is not altered to the extent that receptor binding is prevented. On the other hand, if one wishes to enhance the stability of a 23-aa α-helical direct-acting CAP that is predicted to cause membrane disruption by the barrel-stave model, any of above techniques would likely work with the exception of cyclization, which would prevent the peptide from spanning the cellular membrane. However, it is expected that a cyclic peptide would still be able to disrupt cancer cell membranes if membrane disruption occurs by the carpet or toroidal pore model.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

Additional Strategies To Improve CAP Stability

Several other strategies have been suggested to enhance the stability of CAPs. Oh *et al.* synthesized unnatural aa that are more positively-charged and bulkier than those that occur in nature (*107*). Although these unnatural aa generally increase CAP stability in the presence of serum, the novel aa had a lower α-helical propensity than lysine, which may negatively impact on the biological activity of CAP containing these unnatural aa. CAP stability may also be enhanced by alkylating or acylating lysine residues (*108*, *109*). Since lysine residues are substrates for trypsin digestion, this strategy may interfere with protease recognition of the CAP.

Another strategy to enhance CAP stability is to generate a peptoid equivalent. Peptoids are molecules that are thought to mimic the biological activity of CAP while exhibiting enhanced resistance to proteases owing to the side-chain being positioned on the nitrogen atom rather than the chiral (α) carbon (*110*). Some studies that evaluated the anti-bacterial properties of peptoids used cancer cell lines to test for activity against eukaryotic cells (*111*, *112*). However, a comparison of the *in vitro* cytotoxic activity of peptides and their peptoid equivalent against a panel of cancer cell lines and normal cells has not yet been conducted.

Finally, peptide stability can be increased by forming retro-inverso-isomers, which are directional and chiral isomers of linear peptides (*113*). These pseudopeptides are expected to be resistant to protease-mediated degradation as a result of the change in chirality at the α-carbon. Additionally, these peptide-mimicking structures are predicted to adopt the same side-chain topology as the native peptide; therefore, the biological activity is likely to be retained. All of these modification strategies are novel ways to solve the same problem –

enhanced stability with a concomitant maintenance or enhancement of biological activity. However, to date the impact of peptidomimetics on anti-cancer activity and normal biological responses has not been well studied.

Conclusions

The investigation of the anti-cancer properties of CAPs is still in its infancy, and barriers such as safety and stability have to be overcome before these peptides can be fully evaluated in terms of their potential for use in cancer treatment. Toxicity of membrane-permeabilizing CAPs can be problematic, especially when less selective peptides are employed; however, recent studies show that cancer cell selectivity of CAPs can be enhanced by cautious modification of the peptide's primary aa sequence. Increasing the specificity of these CAPs for cancer cells will decrease the potential for treatment-related toxicities. Furthermore, peptide stability can be enhanced by a variety of strategies, including D-aa incorporation, end capping, and cyclization, all of which need to be considered on an individual basis for any given CAP. Although short peptides can be synthesized at relatively low cost, the production of modified peptides may be more expensive. Nevertheless, the cost of producing peptide-based therapies may be further reduced by generating truncated CAPs that remain capable of mediating selective cancer cell death.

Evidence from *in vitro* and *in vivo* studies indicate that CAPs show considerable promise as novel anti-cancer agents, and are of particular interest because of their predicted ability to kill slow-growing, as well as multi-drug resistant cancers without harming healthy cells and tissues (Table I). The current limitations of CAP-based treatment of cancer are being addressed by using different modification techniques to improve the clinical utility of CAPs. Time and effort will reveal whether there is a place in the clinical setting for cytotoxic peptides in the treatment of cancer.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

References

1. Bulet, P.; Stocklin, R.; Menin, L. *Immunol. Rev.* **2004**, *198*, 169–84.
2. Mader, J. S.; Hoskin, D. W. *Expert Opin. Invest. Drugs* **2006**, *15* (8), 933–46.
3. Hoskin, D. W.; Ramamoorthy, A. *Biochim. Biophys. Acta* **2008**, *1778* (2), 357–75.
4. Yeaman, M. R.; Yount, N. Y. *Pharmacol. Rev.* **2003**, *55* (1), 27–55.
5. Bhutia, S. K.; Maiti, T. K. *Trends Biotechnol.* **2008**, *26* (4), 210–7.
6. Powers, J. P.; Hancock, R. E. *Peptides* **2003**, *24* (11), 1681–91.
7. Silva, P. I., Jr.; Daffre, S.; Bulet, P. *J. Biol. Chem.* **2000**, *275* (43), 33464–70.
8. Rozek, T.; Wegener, K. L.; Bowie, J. H.; Olver, I. N.; Carver, J. A.; Wallace, J. C.; Tyler, M. J. *Eur. J. Biochem.* **2000**, *267* (17), 5330–41.
9. Patrzykat, A.; Friedrich, C. L.; Zhang, L.; Mendoza, V.; Hancock, R. E. *Antimicrob. Agents Chemother.* **2002**, *46* (3), 605–14.
10. Chattopadhyay, S.; Sinha, N. K.; Banerjee, S.; Roy, D.; Chattopadhyay, D.; Roy, S. *Proteins* **2006**, *64* (2), 524–31.

11. Gunshefski, L.; Mannis, M. J.; Cullor, J. S.; Schwab, I. R.; Jaynes, J.; Smith, W. L.; Mabry, E.; Murphy, C. J. *Cornea* **1994**, *13* (3), 237–42.
12. Schwab, U.; Gilligan, P.; Jaynes, J.; Henke, D. *Antimicrob. Agents Chemother.* **1999**, *43* (6), 1435–40.
13. Ma, J.; Kennedy-Stoskopf, S.; Jaynes, J. M.; Thurmond, L. M.; Tompkins, W. A. *J. Virol.* **2002**, *76* (19), 9952–61.
14. Lopez-Garcia, B.; Lee, P. H.; Yamasaki, K.; Gallo, R. L. *J. Invest. Dermatol.* **2005**, *125* (1), 108–15.
15. Aley, S. B.; Zimmerman, M.; Hetsko, M.; Selsted, M. E.; Gillin, F. D. *Infect. Immun.* **1994**, *62* (12), 5397–403.
16. Jaynes, J. M.; Burton, C. A.; Barr, S. B.; Jeffers, G. W.; Julian, G. R.; White, K. L.; Enright, F. M.; Klei, T. R.; Laine, R. A. *FASEB J.* **1988**, *2* (13), 2878–83.
17. Arrowood, M. J.; Jaynes, J. M.; Healey, M. C. *Antimicrob. Agents Chemother.* **1991**, *35* (2), 224–7.
18. Barr, S. C.; Rose, D.; Jaynes, J. M. *J. Parasitol.* **1995**, *81* (6), 974–8.
19. Ballweber, L. M.; Jaynes, J. E.; Stamm, W. E.; Lampe, M. F. *Antimicrob. Agents Chemother.* **2002**, *46* (1), 34–41.
20. Lehmann, J.; Retz, M.; Sidhu, S. S.; Suttmann, H.; Sell, M.; Paulsen, F.; Harder, J.; Unteregger, G.; Stockle, M. *Eur. Urol.* **2006**, *50* (1), 141–7.
21. Eliassen, L. T.; Berge, G.; Leknessund, A.; Wikman, M.; Lindin, I.; Lokke, C.; Ponthan, F.; Johnsen, J. I.; Sveinbjornsson, B.; Kogner, P.; Flaegstad, T.; Rekdal, O. *Int. J. Cancer* **2006**, *119* (3), 493–500.
22. Liberio, M. S.; Joanitti, G. A.; Azevedo, R. B.; Cilli, E. M.; Zanotta, L. C.; Nascimento, A. C.; Sousa, M. V.; Pires Junior, O. R.; Fontes, W.; Castro, M. S. *Amino Acids* **2011**, *40* (1), 51–9.
23. Mader, J. S.; Salsman, J.; Conrad, D. M.; Hoskin, D. W. *Mol. Cancer Ther.* **2005**, *4* (4), 612–24.
24. Rodrigues, E. G.; Dobroff, A. S.; Cavarsan, C. F.; Paschoalin, T.; Nimrichter, L.; Mortara, R. A.; Santos, E. L.; Fazio, M. A.; Miranda, A.; Daffre, S.; Travassos, L. R. *Neoplasia* **2008**, *10* (1), 61–8.
25. Chang, W. T.; Pan, C. Y.; Rajanbabu, V.; Cheng, C. W.; Chen, J. Y. *Peptides* **2011**, *32* (2), 342–52.
26. Jaynes, J. M.; Julian, G. R.; Jeffers, G. W.; White, K. L.; Enright, F. M. *Pept. Res.* **1989**, *2* (2), 157–60.
27. Robertson, C. N.; Roberson, K. M.; Pinero, A.; Jaynes, J. M.; Paulson, D. F. *World J. Urol.* **1998**, *16* (6), 405–9.
28. Arlotti, J. A.; Cimino, T. S.; Nguyen, T.; Dhir, R.; Thomas, A.; Jaynes, J. M.; Caldwell, A. L.; Getzenberg, R. H. *Urol. Oncol.* **2001**, *6* (3), 97–102.
29. Yates, C.; Sharp, S.; Jones, J.; Topps, D.; Coleman, M.; Aneja, R.; Jaynes, J.; Turner, T. *Biochem. Pharmacol.* **2011**, *81* (1), 104–10.
30. Hilchie, A. L. a. H., D. W., The application of cationic antimicrobial peptides in cancer treatment: laboratory investigations and clinical potential. In *Emerging Cancer Therapy: Microbial Approaches and Biotechnological Tools*; Chakrabarty, A., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2010; pp 309−332.
31. Giuliani, A.; Pirri, G.; Nicoletto, S. F. *Cent Eur J Biol* **2007**, *2* (1), 1–33.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

32. Papo, N.; Shai, Y. *Cell Mol. Life Sci.* **2005**, *62* (7-8), 784–90.
33. Bradshaw, J. *BioDrugs* **2003**, *17* (4), 233–40.
34. Baumann, G.; Mueller, P. *J. Supramol. Struct.* **1974**, *2* (5-6), 538–57.
35. Gazit, E.; Boman, A.; Boman, H. G.; Shai, Y. *Biochemistry* **1995**, *34* (36), 11479–88.
36. Shai, Y. *Biochim. Biophys. Acta* **1999**, *1462* (1-2), 55–70.
37. Huang, H. W. *Biochemistry* **2000**, *39* (29), 8347–52.
38. Ludtke, S. J.; He, K.; Heller, W. T.; Harroun, T. A.; Yang, L.; Huang, H. W. *Biochemistry* **1996**, *35* (43), 13723–8.
39. van Beek, W. P.; Smets, L. A.; Emmelot, P. *Cancer Res..* **1973**, *33* (11), 2913–22.
40. Bafna, S.; Kaur, S.; Batra, S. K. *Oncogene* **2010**, *29* (20), 2893–904.
41. Koo, C. Y.; Sen, Y. P.; Bay, B. H.; Yip, G. W. *Recent Pat. Anti-Cancer Drug Discovery* **2008**, *3* (3), 151–8.
42. Utsugi, T.; Schroit, A. J.; Connor, J.; Bucana, C. D.; Fidler, I. J. *Cancer Res..* **1991**, *51* (11), 3062–6.
43. Donnelly, J. G. *Ther. Drug Monit.* **2004**, *26* (2), 231–5.
44. Naumov, G. N.; Townson, J. L.; MacDonald, I. C.; Wilson, S. M.; Bramwell, V. H.; Groom, A. C.; Chambers, A. F. *Breast Cancer Res.. Treat.* **2003**, *82* (3), 199–206.
45. Bush, J. A.; Li, G. *Int. J. Cancer* **2002**, *98* (3), 323–30.
46. Hui, L.; Leung, K.; Chen, H. M. *AntiCancer Res..* **2002**, *22* (5), 2811–6.
47. Johnstone, S. A.; Gelmon, K.; Mayer, L. D.; Hancock, R. E.; Bally, M. B. *Anticancer Drug Des.* **2000**, *15* (2), 151–60.
48. Kim, S.; Kim, S. S.; Bang, Y. J.; Kim, S. J.; Lee, B. J. *Peptides* **2003**, *24* (7), 945–53.
49. Ghavami, S.; Asoodeh, A.; Klonisch, T.; Halayko, A. J.; Kadkhoda, K.; Kroczak, T. J.; Gibson, S. B.; Booy, E. P.; Naderi-Manesh, H.; Los, M. *J. Cell Mol. Med.* **2008**, *12* (3), 1005–22.
50. Ohsaki, Y.; Gazdar, A. F.; Chen, H. C.; Johnson, B. E. *Cancer Res..* **1992**, *52* (13), 3534–8.
51. Zitzmann, S.; Ehemann, V.; Schwab, M. *Cancer Res.* **2002**, *62* (18), 5139–43.
52. Hansel, W.; Enright, F.; Leuschner, C. *Mol. Cell Endocrinol.* **2007**, *260-262*, 183–9.
53. Leuschner, C.; Enright, F. M.; Gawronska, B.; Hansel, W. *Breast Cancer Res. Treat.* **2003**, *78* (1), 17–27.
54. Jia, L.; Noker, P. E.; Piazza, G. A.; Leuschner, C.; Hansel, W.; Gorman, G. S.; Coward, L. U.; Tomaszewski, J. *J Pharm. Pharmacol.* **2008**, *60* (11), 1441–8.
55. Chen, Y.; Xu, X.; Hong, S.; Chen, J.; Liu, N.; Underhill, C. B.; Creswell, K.; Zhang, L. *Cancer Res.* **2001**, *61* (6), 2434–8.
56. Hancock, R. E.; Sahl, H. G. *Nat. Biotechnol.* **2006**, *24* (12), 1551–7.
57. Dennison, S. R.; Whittaker, M.; Harris, F.; Phoenix, D. A. *Curr. Protein Pept. Sci.* **2006**, *7* (6), 487–99.
58. Yang, N.; Strom, M. B.; Mekonnen, S. M.; Svendsen, J. S.; Rekdal, O. *J. Pept. Sci.* **2004**, *10* (1), 37–46.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

59. Makovitzki, A.; Fink, A.; Shai, Y. *Cancer Res.* **2009**, *69* (8), 3458–63.
60. Eliassen, L. T.; Berge, G.; Sveinbjornsson, B.; Svendsen, J. S.; Vorland, L. H.; Rekdal, O. *Anticancer Res.* **2002**, *22* (5), 2703–10.
61. Furlong, S. J.; Mader, J. S.; Hoskin, D. W. *Exp. Mol. Pathol.* **2010**, *88* (3), 371–5.
62. Papo, N.; Braunstein, A.; Eshhar, Z.; Shai, Y. *Cancer Res.* **2004**, *64* (16), 5779–86.
63. Schibli, D. J.; Epand, R. F.; Vogel, H. J.; Epand, R. M. *Biochem. Cell Biol.* **2002**, *80* (5), 667–77.
64. Nilsson, I.; Saaf, A.; Whitley, P.; Gafvelin, G.; Waller, C.; von Heijne, G. *J. Mol. Biol.* **1998**, *284* (4), 1165–75.
65. Chi, S. W.; Kim, J. S.; Kim, D. H.; Lee, S. H.; Park, Y. H.; Han, K. H. *Biochem. Biophys. Res. Commun.* **2007**, *352* (3), 592–7.
66. Dathe, M.; Nikolenko, H.; Meyer, J.; Beyermann, M.; Bienert, M. *FEBS Lett.* **2001**, *501* (2-3), 146–50.
67. Park, Y.; Lee, D. G.; Jang, S. H.; Woo, E. R.; Jeong, H. G.; Choi, C. H.; Hahm, K. S. *Biochim. Biophys. Acta* **2003**, *1645* (2), 172–82.
68. Bellamy, W. R.; Wakabayashi, H.; Takase, M.; Kawase, K.; Shimamura, S.; Tomita, M. *J. Appl. Bacteriol.* **1993**, *75* (5), 478–84.
69. Vorland, L. H.; Ulvatne, H.; Andersen, J.; Haukland, H.; Rekdal, O.; Svendsen, J. S.; Gutteberg, T. J. *Scand. J. Infect. Dis.* **1998**, *30* (5), 513–7.
70. Yang, N.; Stensen, W.; Svendsen, J. S.; Rekdal, O. *J. Pept. Res.* **2002**, *60* (4), 187–97.
71. Strom, M. B.; Rekdal, O.; Svendsen, J. S. *J. Pept. Res.* **2000**, *56* (5), 265–74.
72. Tannock, I. F.; Rotin, D. *Cancer Res.* **1989**, *49* (16), 4373–84.
73. Leuschner, C.; Hansel, W. *Curr. Pharm. Des.* **2004**, *10* (19), 2299–310.
74. Shadidi, M.; Sioud, M. *Drug Resist. Updates* **2003**, *6* (6), 363–71.
75. Grundker, C.; Gunthert, A. R.; Westphalen, S.; Emons, G. *Eur. J. Endocrinol.* **2002**, *146* (1), 1–14.
76. Hansel, W.; Leuschner, C.; Enright, F. *Mol. Cell Endocrinol.* **2007**, *269* (1-2), 26–33.
77. Svenson, J.; Stensen, W.; Brandsdal, B. O.; Haug, B. E.; Monrad, J.; Svendsen, J. S. *Biochemistry* **2008**, *47* (12), 3777–88.
78. Avrahami, D.; Shai, Y. *J. Biol. Chem.* **2004**, *279* (13), 12277–85.
79. Lockwood, N. A.; Haseman, J. R.; Tirrell, M. V.; Mayo, K. H. *Biochem. J.* **2004**, *378* (Pt 1), 93–103.
80. Zweytick, D.; Pabst, G.; Abuja, P. M.; Jilek, A.; Blondelle, S. E.; Andra, J.; Jerala, R.; Monreal, D.; Martinez de Tejada, G.; Lohner, K. *Biochim. Biophys. Acta* **2006**, *1758* (9), 1426–35.
81. Mayo, K. H.; Haseman, J.; Young, H. C.; Mayo, J. W. *Biochem. J.* **2000**, *349* (Pt 3), 717–28.
82. Oren, Z.; Shai, Y. *Eur. J. Biochem.* **1996**, *237* (1), 303–10.
83. Yang, S. T.; Shin, S. Y.; Hahm, K. S.; Kim, J. I. *Int. J. Antimicrob. Agents* **2006**, *27* (4), 325–30.
84. Bernkop-Schnurch, A.; Schmitz, T. *Curr. Drug Metab.* **2007**, *8* (5), 509–17.
85. Rozek, A.; Powers, J. P.; Friedrich, C. L.; Hancock, R. E. *Biochemistry* **2003**, *42* (48), 14130–8.

86. Nguyen, L. T.; Schibli, D. J.; Vogel, H. J. *J. Pept. Sci.* **2005**, *11* (7), 379–89.
87. Conlan, B. F.; Gillon, A. D.; Craik, D. J.; Anderson, M. A. *Biopolymers* **2010**, *94* (5), 573–83.
88. Lehrer, R. I.; Ganz, T. *Ann. N. Y. Acad. Sci.* **1996**, *797*, 228–39.
89. Hoek, K. S.; Milne, J. M.; Grieve, P. A.; Dionysius, D. A.; Smith, R. *Antimicrob. Agents Chemother.* **1997**, *41* (1), 54–9.
90. Tamamura, H.; Ikoma, R.; Niwa, M.; Funakoshi, S.; Murakami, T.; Fujii, N. *Chem. Pharm. Bull. (Tokyo)* **1993**, *41* (5), 978–80.
91. Matsuzaki, K.; Nakayama, M.; Fukui, M.; Otaka, A.; Funakoshi, S.; Fujii, N.; Bessho, K.; Miyajima, K. *Biochemistry* **1993**, *32* (43), 11704–10.
92. Liu, S.; Zhou, L.; Li, J.; Suresh, A.; Verma, C.; Foo, Y. H.; Yap, E. P.; Tan, D. T.; Beuerman, R. W. *ChemBioChem* **2008**, *9* (6), 964–73.
93. Coffelt, S. B.; Scandurro, A. B. *Cancer Res.* **2008**, *68* (16), 6482–5.
94. King, T. P.; Wade, D.; Coscia, M. R.; Mitchell, S.; Kochoumian, L.; Merrifield, B. *J. Immunol.* **1994**, *153* (3), 1124–31.
95. Hedstrom, L. *Chem. Rev.* **2002**, *102* (12), 4501–24.
96. Hedstrom, L.; Perona, J. J.; Rutter, W. J. *Biochemistry* **1994**, *33* (29), 8757–63.
97. Richardson, A.; de Antueno, R.; Duncan, R.; Hoskin, D. W. *Biochem. Biophys. Res. Commun.* **2009**, *388* (4), 736–41.
98. Berge, G.; Eliassen, L. T.; Camilio, K. A.; Bartnes, K.; Sveinbjornsson, B.; Rekdal, O. *Cancer Immunol. Immunother.* **2010**, *59* (8), 1285–94.
99. Hamamoto, K.; Kida, Y.; Zhang, Y.; Shimizu, T.; Kuwano, K. *Microbiol. Immunol.* **2002**, *46* (11), 741–9.
100. Papo, N.; Seger, D.; Makovitzki, A.; Kalchenko, V.; Eshhar, Z.; Degani, H.; Shai, Y. *Cancer Res.* **2006**, *66* (10), 5371–8.
101. Baker, M. A.; Maloy, W. L.; Zasloff, M.; Jacob, L. S. *Cancer Res.* **1993**, *53* (13), 3052–7.
102. Papo, N.; Shahar, M.; Eisenbach, L.; Shai, Y. *J. Biol. Chem.* **2003**, *278* (23), 21018–23.
103. Vajda, T.; Szabo, T. *Acta Biochim. Biophys. Acad. Sci. Hung.* **1976**, *11* (4), 287–94.
104. Jung, H. J.; Park, Y.; Sung, W. S.; Suh, B. K.; Lee, J.; Hahm, K. S.; Lee, D. G. *Biochim. Biophys. Acta* **2007**, *1768* (6), 1400–5.
105. Wang, P.; Gyllner, G.; Kvist, S. *J. Immunol.* **1996**, *157* (1), 213–20.
106. Mader, J. S.; Smyth, D.; Marshall, J.; Hoskin, D. W. *Am. J. Pathol.* **2006**, *169* (5), 1753–66.
107. Oh, J. E.; Lee, K. H. *Bioorg. Med. Chem.* **1999**, *7* (12), 2985–90.
108. Pethe, K.; Bifani, P.; Drobecq, H.; Sergheraert, C.; Debrie, A. S.; Locht, C.; Menozzi, F. D. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (16), 10759–64.
109. Radzishevsky, I. S.; Rotem, S.; Bourdetsky, D.; Navon-Venezia, S.; Carmeli, Y.; Mor, A. *Nat. Biotechnol.* **2007**, *25* (6), 657–9.
110. Mas-Moruno, C.; Cruz, L. J.; Mora, P.; Francesch, A.; Messeguer, A.; Perez-Paya, E.; Albericio, F. *J. Med. Chem.* **2007**, *50* (10), 2443–9.
111. Song, Y. M.; Park, Y.; Lim, S. S.; Yang, S. T.; Woo, E. R.; Park, I. S.; Lee, J. S.; Kim, J. I.; Hahm, K. S.; Kim, Y.; Shin, S. Y. *Biochemistry* **2005**, *44* (36), 12094–106.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

112. Chongsiriwatana, N. P.; Patch, J. A.; Czyzewski, A. M.; Dohm, M. T.; Ivankin, A.; Gidalevitz, D.; Zuckermann, R. N.; Barron, A. E. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (8), 2794–9.

113. Chorev, M.; Goodman, M. *Trends Biotechnol.* **1995**, *13* (10), 438–45.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch004

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

Chapter 5

Lytic Peptides as Anticancer Therapeutics: Lessons Learned from a Novel Design Approach

Clayton Yates,*,[1] Timothy Turner,[1] and Jesse M. Jaynes*,[2]

[1]**Departments of Biology, Center for Cancer Research,**
Carver Research Foundation
[2]**College of Agriculture, Environmental and Natural Sciences**
and School of Veterinary Medicine, Tuskegee University,
Tuskegee Institute, AL 36088
***E-mail: cyates@mytu.tuskegee.edu or jjaynes@mytu.tuskegee.edu**

Lytic peptides represent a novel class of therapeutics that from our view, have not received much attention clinically for the treatment of common cancers. This is partly due to the lack of tumor specificity of these compounds compared with other classes of therapies which enhances the possibilility of unwanted side effects. Herein we detail the use of lytic peptides from their initial use as anti-bacterical/anti-fungal agents to now prospective cancer therapeutic agents. Additionally, we describe a novel design approach that facilitates the modulation of physical peptide characteristics and how this translates lytic activity.

Introduction

According to the American Cancer Society (1 in 2 men) and (1 in 3 females) have a chance of developing cancer in the United States. Furthermore, in 2010 alone about 571,950 Americans are expected to die of cancer (American Cancer Society). These morbidity and mortality rates are mainly attributable to metastasis, as current therapies for localized tumors are not curative and prolong survival by only a few years. Additionally, many tumors of patients who appeared to be cured by surgery reoccur years later due to prior cell dissemination from the primary site (*1*, *2*). Therefore drugs that are effective on primary as well as metastatic tumors

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

are needed. One class of therapeutics that has been often overlooked in cancer therapies are small peptides that have lytic or membrane disrupting functions. In this review we will discuss a class of lytic peptides that have shown effectiveness in several cancer types, and detail the peptide generation, tumor effectiveness, and progress toward clinical use.

Lytic peptides are small proteins that are major components of the antimicrobial defense systems of numerous species. They are a ubiquitous feature of nearly all multi-cellular and some single-cellular life forms. Generally consisting of between 10-40 amino acid sequences, lytic peptides have the potential for forming discrete secondary structures. Often, they exhibit the property of amphipathy i.e. the segregation and concentration of nonpolar and polar amino acids on opposite sides and along the length of the molecule (*3*).

H.G. Boman and colleagues (*4*) were the first to clearly describe the humoral defense system utilized by *Hyalophora cecropia,* the giant silk moth, as a protective mechanism against bacterial infection. Their work, along with that of Lehrer and collaborators (*5*) in characterizing the human defensins, stand as models for the delineation of this type of ubiquitous natural immune-protection in living organisms. Boman's group discovered unique proteins in the insect's hemolymph after induction by either live or heat-killed bacteria that were capable of membrane perturbation resulting in bacterial cell lysis. Among this family of inductive proteins were a type designated as the cecropins (*6*). The three principal cecropins: A, B, and D, are highly homologous (*7*), small basic proteins each containing a comparatively long hydrophobic region. Their primary mode of action was membrane disruption and subsequent lysis due to the target cell 's loss of osmotic integrity (*8*). Several years later, similar types of lytic proteins were shown to play key roles in providing protection from disease in other organisms. For example, peptides isolated from amphibians by Gibson and co-workers (*9*) and Giovannini and co-workers (*10*), and independently by Zasloff (*11*), all possess antibacterial activity.

While the antibacterial effect of lytic peptides from insects and amphibians had been well documented, there were no published reports of their potential effectiveness against mammalian cells At the time, it was assumed that these types of peptides were limited to antibacterial activity only. However, our work has described a number of instances that this was not the case, as indeed lytic peptides have activity on number of cell types including, fungi, protozoa, viruses and transformed cells (*12–15*).

Lytic Peptide Design Principles

Most of the α-helical lytic peptides that have been described in the literature fall into one of three different classes based on the arrangement of amphipathy and high positive charge density within the molecule (*16*). Each class possesses an array of physical features that establish the uniqueness of the peptide's class. There are a number of physical features that play a role in modulating the activity of these types of peptides, including degree and length of amphipathy, hydrophobicity, and surface area of both hydrophilic and hydrophobic faces.

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

These individual physical characteristics can be distinguished by analyzing successive amino acids sequences in a format that attenuates the individual contributing physical properties in a three-dimensional format. Given that there are only 20 different amino acids found in a given protein, is it tempting to speculate the evolution of protein structure around these basic characteristics.

To facilitate exploration of the depth of plasticity of the structure/function paradigm of designed lytic peptides, a very simple method, a molecular font call "Molly", was devised more than 15 years ago that allowed illustration of the physical connections existing between lytic peptides ((*16*) also See Rajasekaran's chapter (Chapter 14) in this volume). Utilizing, *Molly*, we have performed a series of design manipulations of naturally occurring and synthetically designed lytic peptides with an emphasis on retaining thea characteristically positively charged and potentially amphipathic alpha-helical or beta-pleated sheet structure (*17*).

In Vitro Activity of Designed Lytic Peptides Against Transformed Mammalian Cells

The first paper to appear in the literature (in 1989) described the *in vitro* cytocidal effect of three synthetic lytic peptides on several transformed mammalian cell lines (*18*). Two of the peptides were closely related analogs of cecropin B (SB-37 and SB-37*) containing relatively minor changes in amino acid sequence from the native cecropin B peptide, while the other was a distinct peptide, Shiva-1, designed to have significant differences in sequence homology, while conserving the overall charge distribution and hydrophobic properties of the natural cecropin B molecule (*19*). The enhanced bioactivity of Shiva-1 was the first indication that modifications made in the primary sequence of lytic peptides would not destroy the peptide's activity, provided certain physical characteristics of the peptide were conserved. Indeed, this was a paradigm-shifting moment in understanding of the structure/function relationship of these incredibly interesting natural molecules and allowed us to pursue the design of novel molecules with enhanced activities. Another example of sequence modification resulting in increased potency of natural peptides is provided in MSI-99, an analog of magainin-II that displayed more positive charge and antibacterial and antifungal activity than its predecessor (*20*). These characteristics indicate that the specific amino acid sequence of the lytic peptide is irrelevant to peptide function as long as certain physical properties of the peptide are maintained.

It was well known then that many transformed or cancerous cells undergo epithelial-to-mesenchymal transition (EMT) that is associated with a much less organized cytoskeleton than normal epithelial counterparts. (*21*, *22*). One of the hallmarks of the aggressive mesenchymal cells is the lack of cohesiveness , which fosters increased migratory and invasive properties and a less stable cellular membrane (*23*). Thus, the membrane perturbation properties of lytic peptides, creates a plausible situation where aberrant cells may be more sensitive. To test this hypothesis we utilized several transformed cell lines, of various cancer types, and exposed several novel peptide designs to ascertain their lytic activity. We found that most peptide designs were equally active in lysing the cancer cells at the approximate concentrations as reported in bacteria. In all cases, the peptides caused an increase in cytotoxicity as measured by ^{51}Cr release. SB-37 and SB-37*, the derivatives which most closely resembled the native cecropin B molecule in terms of amino acid sequence homology, were less active than Shiva-l, the lytic peptide which departed the most from the natural sequence (*3*). Shiva-l retained about 40% sequence homology, but the overall charge density and hydrophobic/hydrophilic profile of cecropin B had been maintained. Merrifield and colleagues had demonstrated that single amino acid substitutions, made in cecropin A drastically reduced its lytic effect in bacteria (*24*). However, these changes made in cecropin A did not conserve charge density nor maintain the native molecule's hydrophobic/hydrophilic profile, those biochemical properties are requisite for normal biological activity (*25*).

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

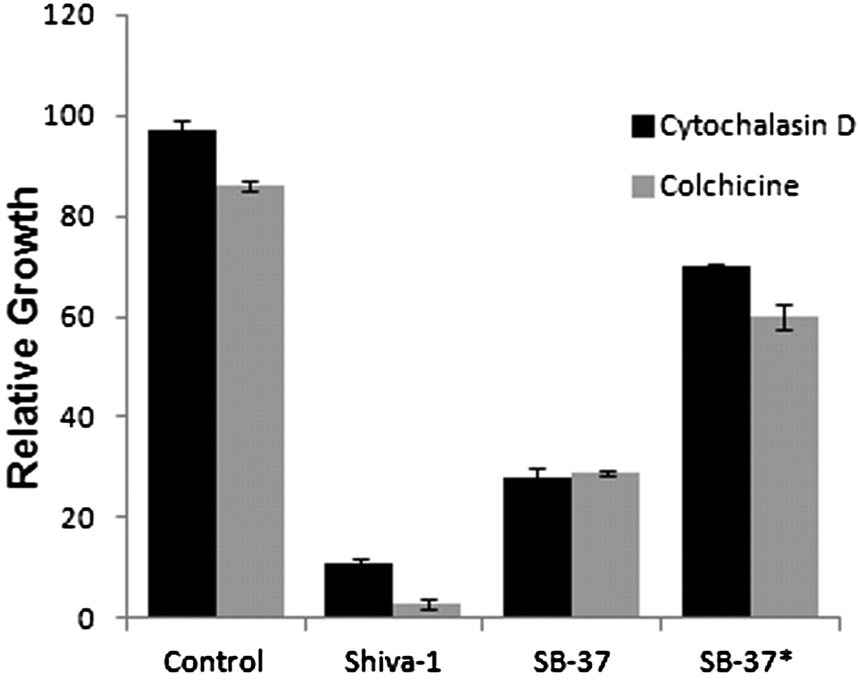


Figure 1. ***Sensitivity to lysis of normal fibroblasts by lytic peptides when pretreated with cytochalasin D and colchicine.*** *A highly significant reduction in cell viability was observed in normal fibroblasts (donkey dermal), as measured by try pan blue exclusion, when these cells were pretreated with the known cytoskeletal inhibitors cytochalasin D (at 5 µg/µl) and colchicine (at 10 µM). Results are means ± SE of three independent experiments.*

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

Table I. Sequence of Set of Designed Lytic Peptides. The single letter amino acid code of a number of designed lytic peptides with their hydrophobic tails underlined

1A	FAVAVKAVKKAVKKVKKAVKKAVKKKK
1D	FAVAVKAVKKAVKKVKKAVKKAV
2A	KKKKFVKKVAKKVKKVAKKVAKVAVAV
2D	FVKKVAKKVKKVAKKVAKVAVAV
3A	KKKKFVKKVAKVAKKVAKVAKKVAKKV
3D	FVKKVAKVAKKVAKVAKKVAKKV
3G	FVKKVAKVAKKVAKVAKKVAKKVKKKK
4B	FKVKAKVKAKVKAKVKAKKKK
4E	FKVKAKVKAKVKAKVKA
4H	KKKKFKVKAKVKAKVKAKVKA
5A	FAVGLRAIKRALKKLRRGVRKVAKRKR
5B	FAVGLRAIKRALKKLRRGVRKVA
5C	KRKRAVKRVGRRLKKLARKIARLGVAF
5D	AVKRVGRRLKKLARKIARLGVAF
5E	FAVGLRAIKRALKKLRRGVRKVAKRKRKDL
5F	FAVGLRAIKRALKKLRRGVRKVAKDL
5G	KRKRAVKRVGRRLKKLARKIARLGVAFKDL
5H	AVKRVGRRLKKLARKIARLGVAFKDL
1A6	FALALKALKKALKKLKKALKKAL
1A6M	MFALALKALKKALKKLKKALKKAL
1A21	FAFAFKAFKKAFKKFKKAFKKAF
1A4	FAIAIKAIKKAIKKIKKAIKKAI
2A21	FAKKFAKKFKKFAKKFAKFAFAF
4E1	FKLRAKIKVRLRAKIKL
5C1	KRKRAVKRVGRRLKKLARKIARLGVAKLAGLRAVKLF
1A6E	FALALKALKKALKKLKKALKKALKDL

To determine why cancer cell lines exhibited an increased tendency to lyse in the presence of lytic peptides over normal mammalian cells, we next sought to determine if normal adherent mammalian cells could be induced become sensitive to cellular lysis. Since it appeared that a well-developed cytoskeletal system was requisite in conferring resistance to normal cells to the effects exerted by the lytic peptides, we utilized known inhibitors of microtubule and microfilament polymerization (colchicine and cytochalasin D, respectively) to determine if this influenced the level of lytic activity observed in cancer cell lines. As predicted the addition of colchine and cytochalasin D pretreatments, causes normal cells to become similarly sensitive to the effects of the peptides as cancer cell lines (see Figure 1)

These findings confirmed that the action of lytic peptides on normal mammalian cells was rather limited and, for the most part, was not lethal. Furthermore, the resistance to lytic activity was most probably due to the well organized cytoskeletal network characteristic of normal epithelial cells, which aided the cell in maintenance of its general shape and polarity, thus enhancing overall osmotic integrity. Much has been learned since this time on the mechanism of action of lytic peptides on prokaryotic and eukaryotic cells. An excellent discourse on this topic appears in Chapter 4 of this book (Obstacles and Solutions to the Use of Cationic Antimicrobial Peptides in the Treatment of Cancer).

Over the last 25 years more than 30,000 peptides have been designed with close to 400 different sequences of these designs made and tested. Design principles were developed over several sequential steps of design, testing and subsequent design. . From the original Shiva designs, several more iterations were developed Table I.

To determine if these new designs exerted anti-cancer activity, we began testing the anti-neoplastic activity by screening these new designs at low concentrations ranging from 1 nM to 10 μM against in human breast cancer cell lines model T47-D, MDA-231 and BT-474; osteosarcoma line MG-63; nasopharyngeal carcinoma line KBATCC; and murine B16 melanoma. Over the 26 drug design panel all peptides showed significant anti-cancer activity below the 10μM, when the averages of each cell line were combined. However, two compounds 2A21 and 1A21 demonstrated the most effectiveness at the lowest concentrations with LD_{50} values of 2.8 and 2.9 μM, respectively (unpublished observations).

Over the next few years, we and others conducted a number of in vitro studies testing the effectiveness of eliminating a widely disparate range of cancer cell lines including commonly utilized prostate cancer cell lines. We again tested nine novel designed lytic peptides for activity against four androgen-insensitive prostate cancer cell lines using a standard cell proliferation assay: MTT. Five of the peptides were known to form alpha-helical secondary structures and were highly active against prostate cancer cell lines. Three peptides configured in beta-pleated sheets were noticeably less effective. Concentrations lethal to 50% of the prostate-cancer cell lines treated LD_{50} values ranged from 0.6 to 1.8 μM. For comparison, two of the highly active alpha-helically structured peptides, D2A21 and Hecate, were tested on several other cancer types: breast, colon, bladder, cervical and lung carcinomas. LD_{50} values recorded for D2A21 and DP1E in cervical, colon, bladder, and lung cancer lines were similar to those obtained in prostate cancer cells (data not shown). As compared with cisplatin (LD_{50} of 7.01 μM), a standard chemotherapeutic drug, the LD_{50} values recorded for D2A21 were significantly lower ($P < 0.04$) in prostate-cancer cell lines, suggesting the therapeutic efficacy of lytic peptides. These data demonstrated for the first time the cytotoxic potential

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

of designed lytic peptides against prostate cancer and provided proof of principle of the use of these compound for treatment of prostate cancer (*26*).

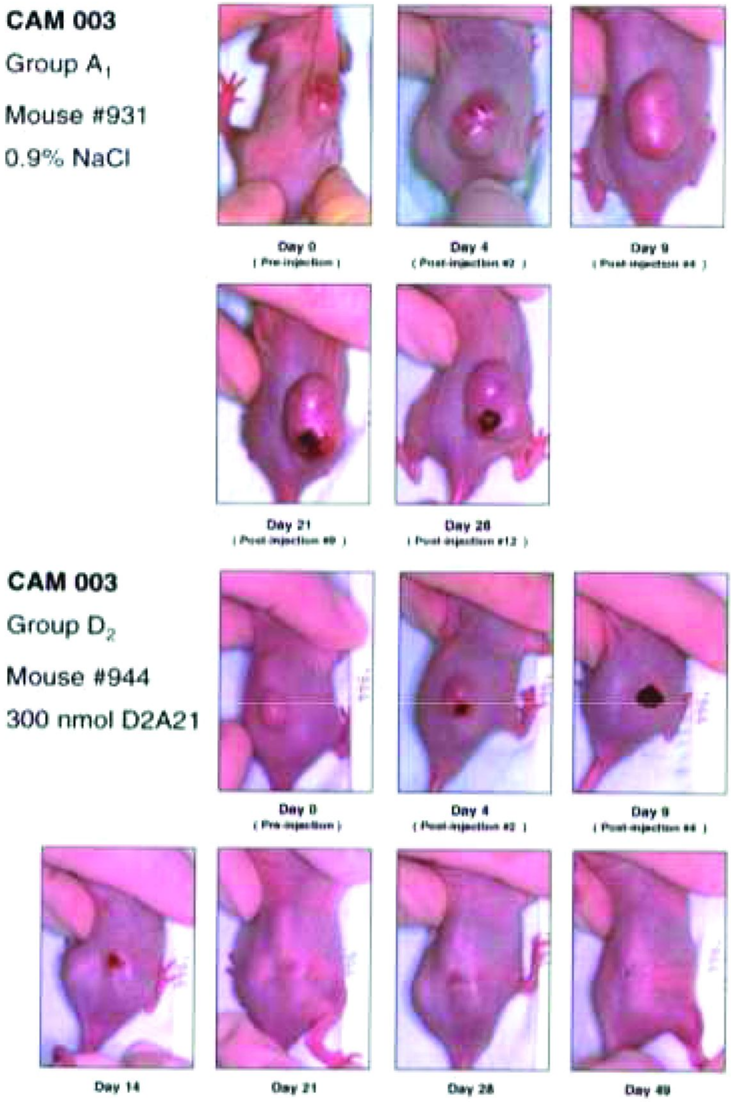

CAM 003
Group A_1
Mouse #931
0.9% NaCl
Day 0
(Pre-injection)
Day 4
(Post-injection #2)
Day 9
(Post-injection #4)
Day 21
(Post-injection #8)
Day 28
(Post-injection #12)
CAM 003
Group D_2
Mouse #944
300 nmol D2A21
Day 0
(Pre-injection)
Day 4
(Post-injection #2)
Day 9
(Post-injection #4)
Day 14
Day 21
Day 28
Day 49

Figure 2. ***D2A21 inhibits human prostate tumor xenograft growth in nude mice.*** *Male nude mice were subcutaneously injected with PC-3 cells . (Top) photographs show that 0.9% NaCl (control) treated tumors demonstrated typical tumor growth with visible angiogensis over identical 49 day time period. (Bottom) demonstrate that 300 nmol of D2A21 treated tumors regressed over a nine day period, and tumor areas were completely absent after 49 days.*

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

In Vivo Activity of Designed Lytic Peptides Against Transformed Mammalian Cells

Since our peptide design algorithm which enhances activity and reduced toxicity was realized in several peptide designs, including D2A21, we next sought to determine the effectiveness of D2A21 on PC-3 xenograft tumors. PC-3 xenografts were chosen because they are hormone resistant and more accurately represent the most aggressive and metastases encountered clinically. D2A21 exhibited time and dose dependent cytotoxic activity in prostate cancer cells in vivo (see Figure 2). Tumor regression was visibly dramatic and resulted in a maximum of 95% necrosis by microscopic examination (Figure 2). Necrosis was seen to a much greater degree in treated tumor tissue than in controls. Treatment with D2A21, at low doses (100-300 nmol per injection), resulted in dose dependent regression of PC-3 tumors in the flanks of athymic mice (data not shown), with 300 nmol displaying the most efficacious dosage. No systemic toxicity was observed after multiple injections of peptide and there was no evidence of tumor recurrence, although high concentration did display toxicity (data not shown). Mice given 300 nmol had the longest average survival, the greatest percentage of tumor regression, and the lowest average number of injections required to achieve successful tumor regression (*27*).

Systemic Anticancer Activity of Lytic Peptides

Since intra-tumoral injection demonstrated significant anti-cancer activity, we next sought to determine if D2A21 administered intra-peritoneally would have similar effects. D2A21 was administered three times each week for three weeks beginning on the same day that the MLL cells (a highly aggressive rat prostate cancer cell line) were injected. Five groups, each consisting of 12–13 rats, received between 0.0357 mg and 7.14 mg of peptide on each injection day. A sixth group of 12 rats served as a control group receiving saline injections three times each week. Exposure to doses of 0.179 mg or more of the D2A21 peptide significantly improved survival by 67%, when animals were given 0.179 mg or more of D2A21. All animals experienced some weight gain, and there did not appear to be a substantial toxicity associated with any of the doses utilized. Lastly, this was repeated multiple times with similar results.

In order to determine the influence of administration of D2A21 on lung metastases in this animal model, a quantitative analysis was performed on 12 lungs from animals receiving subcutaneous injections of the peptide (at 0.179 mg) and 12 lungs from animals receiving subcutaneous saline injections. These studies were performed by first removing and then sectioning the entire lung from each animal. The total lung area was determined utilizing a computer-based digital image analysis system. A larger number of evident metastases were identified as determined b by the tumor number and area. There was a 90% decrease in the number of metastases in the peptide treated group when compared to the saline controls (*28*).

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

Table II. Interpolated Mean IC50 Values Obtained from Prostate Cancer Cell Lines IC50s on LNCaP, DU-145 and PC-3 cells were determined by interpolation of 3 individual experiments perfomed in quadruplicate at 50% cell death. All values were determined to be significant compared to control at p<0.0001. n.d. = not determined

Cell Line	*Peptides*			
	JC21	*JCH*	*JC21LHRH*	*JCHLHRH*
LNCaP	n.d.	n.d.	9.15 µM	4.36 µM
DU-145	n.d.	n.d.	5.66 µM	4.81 µM
PC-3	9.25 µM	6.67 µM	7.42 µM	4.22 µM

Tumor Targeting Lytic Peptides

We along with several other groups, independently have demonstrated both the *in vitro* and *in vivo* efficacy of targeting LHRH receptor directly with receptor agonist (*29*) and antagonist (*30*) or through LHRH-conjugated agents to treate hormonally regulated cancers (*31*, *32*). This is plausible given that LHRH-Rs are expressed in 86% of human prostate cancers and LHRH-R numbers increase with the increasing metastatic potential of prostate cancer cell lines (*33*). Additionally LHRH therapy remains the standard form of treatment for men with metastastic prostate cancer. Thus, targeting LHRH-R presents a clinical target to add tumor-specificity to the our design of anti-cancer lytic peptides.

To develop LHRH-lytic peptide conjugates in our hands, we first sought to modify LHRH 10-amino acid sequence (QHWSYGLRP) with a single amino acid modification (QHWSWGLRP) to increase hydrophobicity and enhance anti-tumor activity while adhering to the strict criteria of limiting activity on non-cancerous cells. This sequence is in constast to LHRH-sequences proposed by other groups (*34–38*). Additionally, new lytic peptide sequences were designed, based on criteria stated above, to further ehance the anti-cancer activity. Two compounds JCH and JC21 were derived (*39*) and tested with or without LHRH conjugates on the highly metastatic PC-3 cells. Both peptides exhibited a dose-dependent decrease in cell proliferation, with JCH and JC21 having IC_{50} values of 6.67 and 9.25 µM, respectively. Surprisingly, the addition of LHRH sequence lowered the IC_{50} concentrations to 4.22 µM for JCHLHRH and 7.24 µM for JC21LHRH (see Table II). JCHLHRH and JC21LHRH were also effective on the androgen-dependent LNCaP and androgen-independent DU-145 cell lines. IC_{50} values for LNCaP cells were 4.36 µM for JCHLHRH and 9.15 µM for JC21LHRH and DU-145 cells 4.81 µM for JCHLHRH and 5.66 µM for JC21LHRH. Furthermore cell death was extremely robust, as we confirmed nearly 80% cell death after only 6 hr of treatment and near 100% cell death after only 24 hr through both real-time imaging and trypan blue staining. As expected both peptides showed minimal effects on normal primary hPrEC cells or bone marrow stromal cells, even at high concentrations, further demonstrating the cancer cell specificity of these compounds. All IC_{50} values are summarized in Table II.

These peptides represent a novel class of cancer therapeutic that from our view, have not received much attention clinicaly. This is impart due to the lack of tumor specificity of these compound compare to other targeted compound design. Although *in vivo* investigations of for this last peptide design in animal cancer model systems are ongoing, we are hopeful for the effectiveness, given the significant tumor activity and lack of toxicity of previous peptide design D2A21. In fact, D2A21 has just successfully completed phase I clinical trials as a cancer treatment. Thus these peptides appear to be eventual candidates for use in the treatment of local and metastatic prostate cancer.

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

Conclusion

In conclusion, these results provide evidence for the use of lytic peptides, in particular D2A21 and tumor targeting JCHLHRH and JC21LHRH, as novel cancer therapeutics. Furthermore, these findings provide proof-of-principle that the design of lytic peptides needs to be further explored and exploited for clinical utility. Although the exact mechanism through which lytic peptides interact with cell membranes is still under investigation, particularly receptor mediated tumor-targeting, these compounds can be administered through a variety of sites, this includes subcutaneous and intra-peritoneal routes, with little or no toxicity at concentrations shown to efficacious against tumors. The work described constitutes a substantial advancement in drug design and delivery and provides promise for suffering cancer patients.

Acknowledgments

The authors would like to acknowledge all collaborators involved in the different phases of this work over the years. The most current work was supported by grants U54 CA118623 (NIH/NCI) to T.T. G12RR03059-22 to PC07397 (Department of Defense) to C.Y.

References

1. Yates, C. Prostate tumor cell plasticity: a consequence of the microenvironment. *Adv. Exp. Med. Biol.* **2010**, *720*, 81–90.
2. Yates, C. C.; Shepard, C. R.; Stolz, D. B.; Wells, A Co-culturing human prostate carcinoma cells with hep.atocytes leads to increased expression of E-cadherin. *Br. J. Cancer* **2007**, *96*, 1246–1252.
3. Jaynes, J. M.; Burton, C. A.; Barr, S. C.; Jeffers, G. W.; Julian, G. R.; White, K. L.; Enright, F. M.; Klei, T. R.; Laine, R. A. In vitro cytocidal effects of novel lytic peptides on Plasmodium falciparum and Trypanosoma cruzi. *FASEB J.* **1988**, *2*, 2878–2883.
4. Hultmark, D.; Steiner, H.; Rasmuson, T.; Boman, H. G. Purification and properties of three inducible bacterial proteins from the hemolymph of

immunized pupae of Hyalophora cecropia. *Eur J. Biochem.* **1980**, *106*, 7–16.

5. Lehrer, R. I.; Lichtenstein, A. K.; Ganz, T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* **1993**, *11*, 105–128.
6. Boman, H. G.; Steiner, H. Humoral immunity in *Cecropia* pupae. *Curr. Top. Microbiol. Immunol.* **1981**, *94/95*, 75–91.
7. Boman, H. G. Antibacterial peptides: basic facts and emerging concepts. *J. Intern. Med.* **2003**, *254*, 197–215.
8. Boman, H. G. Peptide antibiotics and their role in innate immunity. *Annu. Rev. Immunol.* **1995**, *13*, 61–92.
9. Gibson, B. W.; Poulter, L.; Williams, D. H.; Maggio, J. E. Novel peptide fragments originating from PGL and caerulin and xenopsin precursors from Xenopus laevis. *J. Biol. Chem.* **1986**, *261*, 5341–5349.
10. Giovannini, M. G.; Poulter, L.; Gibson, B. W.; Williams, D. W. Biosynthesis and degradation of peptides from *Xenopus laeviis* prohormones. *Biochem. J.* **1987**, *243*, 113–120.
11. Zasloff, M. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449–53.
12. Jaynes, J. M. Lytic peptides: harbingers of a new age in the treatment of disease. *New Sci.* **1989**, 42–44.
13. Schwab, U.; Gilligan, P.; Jaynes, J.; Henke, D. In vitro acitivities of designed antimicrobial peptides against multidrug-resistant cystic fibrosis pathogens. *Antimicrob. Agents Chemother.* **1999**, *43*, 1435–1440.
14. Arrowood, M. J.; Jaynes, J. M.; Healy, M. C. In vitro activities of lytic peptides against the sporozoites of cryptosporidium parvum. *Antimicrob. Agents Chemother.* **1991**, *35*, 224–27.
15. Miller, M. A.; Garry, R. F.; Jaynes, J. M.; Montelaro, R. C. A structural correlation between lentivirus transmembrane proteins and natural cytolytic peptides. *AIDS Res. Hum. Retrov.* **1991**, *7*, 511–19.
16. Rajasekaran, K.; Jaynes, J. M.; Cary, J. W. Transgenic expression of lytic peptides in food and feed crops to control phytopathogens and preharvest mycotoxin contamination mycotoxin prevention and control in agriculture. In *Mycotoxin Prevention and Control in Agriculture*; ACS symposium Series 1031; American Chemical Society: Washington, DC, 2009; pp 119−142.
17. Jaynes, J. M.; Xanthopoulos, D.; Destefano-Beltran, L.; Dodds, J. H. Increasing bacterial disease resistance in plants utilyzing antibacterial genes from insects. *Bioessays* **1987**, *6*, 263–70.
18. Jaynes, J. M.; Julian, G. R.; Jeffers, G. W.; White, K. L.; Enright, F. M. In vitro cytocidal effect of lytic peptides on several transformed mammalian cell lines. *Pept. Res* **1989**, *2* (2), 157–160.
19. Destefano-Beltran, L.; Nagpala, P. G.; Selim Cetiner, M.; Dodds, J. H.; Jaynes, J. M. Enhancing Bacterial and Fungal Disease Resistance in Plants: Application to Potato. *Mol. Biol. Potato* **1990**, 205–221.

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

20. DeGray, G.; Rajasekaran, K.; Smith, F.; Sanford, J.; Daniell, H. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant Physiol.* **2001**, *127*, 852–62.
21. Witkowski, C. M.; Rabinovitz, I.; Nagle, R. B.; Affinito, K. S.; Cress, A. E. Characterization of integrin subunits, cellular adhesion and tumorgenicity of four human prostate cell lines. *J. Cancer Res. Clin. Oncol.* **1993**, *119*, 637–644.
22. Romanov, V.; Goligorsky, M. S. RGD-recognizing integrins mediate interactions of human prostate carcinoma cells with endothelial cells in vitro. *Prostate* **1999**, *39*, 108–118.
23. Cooper, C. R.; Bhatia, J. K.; Muenchen, H. J.; McLean, L.; Hayasaka, S.; Taylor, J.; Poncza, P. J.; Pienta, K. J. The regulation of prostate cancer cell adhesion to human bone marrow endothelial cell monolayers by androgen dihydrotestosterone and cytokines. *Clin. Exp. Metastas.* **2002**, *19*, 25–33.
24. Merrifield, R. B.; Bowman, H. G.; Andreu, D. *In Proceedings of the 21st European Peptide Symposium*; Giralt, E., Andreu, D., Eds.; Platja díAro, Spain, 2ñ8 Sept. ESCOM Science Publishers: Leiden, The Netherlands.1990; pp 3−16.
25. Kelly, D. G.; Wolters, W. R.; Jaynes, J. M.; Newton, J. C. Enhanced disease resistance to enteric septicemia in channel catfish, *lctalurus punctatus*, administered lytic peptide. *J. Appl. Aquacult.* **1993**, *3* (1-2), 25–34.
26. Robertson, C.; Roberson, K.; Pinero, A.; Jaynes, J.; Paulson, D. Peptidyl membrane-interactive molecules are cytotoxic to prostate cancer cells in vitro. *World J. Urol* **1998**, *16*, 405–09.
27. Makhuli, M. J.; Roberson, K. M.; Poulton, S. H. M.; Vollmer, R. T.; Padilla, G. M.; Edwards, D. W.; Yancey, D. R.; Caldwell, A. L; Jaynes, J. M.; Robertson, C. N. In Vivo, Synthetic Lytic Peptides Exhibit Anit-Tumor Activity Against Prostate Cancer. *American Urological Annual Meeting* **1998**.
28. Arlotti, J. A.; Cimino, T. S.; Nguyen, T. S.; Dhir, R.; Thomas, A; Jaynes, J. M.; Caldwell, A. L.; Getzenberg, R. H. Efficacy of a synthetic lytic peptide in the treatment of prostate cancer. *Urol. Oncol.* **2001**, *6*, 97–102.
29. Wells, A.; Souto, J. C.; Solava, J.; Kassis, J.; Bailey, K. J.; Turner, T. Luteinizing hormone-releasing hormone agonist limits DU-145 prostate cancer growth by attenuating epidermal growth factor receptor signaling. *Clin. Cancer Res.* **2002**, *8*, 1251–1257.
30. Yates, C.; Wells, A.; Turner, T. Luteinizing hormone-releasing hormone analogue reverses the cell adhesion profile of EGFR overexpressing DU-145 human prostate carcinoma subline. *Br. J. Cancer* **2005**, *92*, 366–375.
31. Leuschner, C.; Enright, F. M.; Gawronska-Kozak, B.; Hansel, W. Human prostate cancer cells and xenografts are targeted and destroyed through luteinizing hormone releasing hormone receptors. *Prostate* **2003**, *56*, 239–249.
32. Leuschner, C.; Hansel, W. Targeting breast and prostate cancers through their hormone receptors. *Biol. Reprod.* **2005**, *73*, 860–865.

33. Qayum, A.; Gullick, W.; Clayton, R. C.; Sikora, K.; Waxman, J. The effects of gonadotrophin releasing hormone analogues in prostate cancer are mediated through specific tumour receptors. *Br. J. Cancer* **1990**, *62*, 96–99.
34. Bodek, G.; Kowalczyk, A.; Waclawik, A.; Huhtaniemi, I.; Ziecik, AJ Targeted ablation of prostate carcinoma cells through LH receptor using Hecate-CGbeta conjugate: functional characteristic and molecular mechanism of cell death pathway. *Exp. Biol. Med. (Maywood)* **2005**, *230*, 421–428.
35. Hansel, W.; Leuschner, C.; Enright, F. Conjugates of lytic peptides and LHRH or betaCG target and cause necrosis of prostate cancers and metastases. *Mol. Cell. Endocrinol.* **2007**, *269*, 26–33.
36. Hansel, W.; Leuschner, C.; Gawronska, B.; Enright, F. Targeted destruction of prostate cancer cells and xenografts by lytic peptide-betaLH conjugates. *Reprod. Biol.* **2001**, *1*, 20–32.
37. Hansel, W.; Enright, F.; Leuschner, C. Destruction of breast cancers and their metastases by lytic peptide conjugates in vitro and in vivo. *Mol. Cell. Endocrinol.* **2007**, *262*, 183–189.
38. Leuschner, C.; Enright, F. M.; Gawronska, B.; Hansel, W. Membrane disrupting lytic peptide conjugates destroy hormone dependent and independent breast cancer cells in vitro and in vivo. *Breast Cancer Res. Treat.* **2003**, *78*, 17–27.
39. Yates, C.; Sharp, S.; Jones, J.; Topps, D.; Coleman, M.; Aneja, R.; Jaynes, J.; Turner, T. LHRH-conjugated lytic peptides directly target prostate cancer cells. *Biochem. Pharmacol.* **2010**, *81*, 104–110.

Downloaded by STANFORD UNIV GREEN LIBR on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch005

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Chapter 6

Strategies for Designing Peptide Immunogens To Elicit α-Helical Conformation-Specific Antibodies Reactive with Native Proteins

Zhe Yan,[1,†] Wendy J. Hartsock,[1,†] Zhaohui Qian,[2] Kathryn V. Holmes,[2] and Robert S. Hodges*[,1]

[1]Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Aurora, CO 80045, U.S.A.
[2]Department of Microbiology, University of Colorado School of Medicine, Aurora, CO 80045, U.S.A.
***E-mail: Robert.Hodges@ucdenver.edu. Phone: 303-724-3252. Fax: 303-724-3249**
†Equal contributors.

Synthetic peptide vaccines against epitopes from the native proteins of pathogenic organisms have the potential to replace traditional vaccines if they can mimic the structure of the epitope found in the native protein target. We have developed a robust technology to elicit antibodies that recognize α-helical sequences of native proteins using a peptide template that consists of a parallel, two-stranded, α-helical coiled-coil. The surface-exposed residues from a helical sequence of interest are inserted into the template to elicit conformation-specific antibodies that recognize the same sequence in the native protein. This strategy was used to develop a vaccine candidate for the pathogen responsible for a 2003 outbreak of severe acute respiratory syndrome (SARS), the SARS coronavirus (SARS-CoV). The SARS-CoV Spike (S) glycoprotein is a class I viral fusion protein that possesses regions containing hydrophobic heptad repeats (HR) at the C-terminus (HRC) and at the N-terminus (HRN) of the fusion domain of S protein. The coiled-coil structures formed by the HRC and HRN regions undergo a series of conformational changes that ultimately mediate membrane fusion during virus entry

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

and virus transmission between host cells. Three different peptides were designed to display the HRC region as a one-stranded peptide, templated two-stranded coiled-coil or peptide scaffold-three stranded coiled-coil and conjugated to keyhole limpet haemocyanin (KLH) to form the immunogens used to elicit antibodies in rabbits. We prepared three additional constructs to evaluate these antibodies, a stabilized HRC trimer (GCN4-HRC-GCN4 construct to mimic the prefusion conformation of S protein), a less stable and more flexible trimer of HRC (HRC-GCN4) and a third construct comprised of HRC and HRN peptides to form the six-helix bundle of the postfusion conformation of S protein. Even though all three peptide immunogens contained the same HRC sequence, their corresponding antibodies demonstrated a wide range of affinities to the GCN4 constructs, BSA-peptide conjugates and the native S protein and had different virus neutralizing activities. The templated two-stranded HRC peptide had the highest helical content and was the most thermally stable peptide immunogen of the three peptide immunogens examined here. Importantly, the antibody elicited against this peptide was the only antibody capable of binding the prefusion state of the native S protein, preventing virus entry and inhibiting S protein mediated cell-cell fusion. This antibody exhibited the strongest binding to the GCN4 constructs (HRC-GCN4 and GCN4-HRC-GCN4) and had the weakest affinity for the postfusion conformation of HRC (six-helix bundle construct). Our conformation-stabilized two-stranded coiled-coil template acts as an excellent platform to elicit α-helix-specific antibodies against native proteins and can be exploited to develop vaccine candidates against a wide variety of viral pathogens where α-helical regions are important for viral entry. Here, we review techniques to generate effective synthetic peptide immunogens to elicit antibodies that recognize native proteins and present our work targeting SARS-CoV.

Keywords: Synthetic peptide immunogen; alpha helix; conformation-specific antibody; SARS-CoV S protein; two-stranded α-helical coiled-coil template

Introduction to Synthetic Peptide-Based Vaccines

Vaccination is the primary strategy to combat viral infections in humans. This approach has been very successful for control of a wide range of pathogens, such as smallpox, which was eradicated in 1979, polio and measles (*1*). Traditional vaccines are composed of live attenuated microorganisms or inactivated (killed) microorganisms. However, it is not always practical to produce live attenuated

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

or inactivated vaccines since some pathogenic microorganisms are difficult to culture *in vitro*. In addition, some features of attenuated microorganisms may result in detrimental immune responses or specific components of the pathogen in the vaccine may contain material that initiates an unwanted host immunological response (*2*). With the current wealth of knowledge concerning potential vaccine targets, an alternative strategy is to generate recombinant vaccines based on particular protein antigens from the targeted microorganism. The concept of producing subunit vaccines was first proposed three decades ago for influenza (*3*). Although a number of successful vaccines have been generated using correctly folded recombinant proteins, the desired immune response, including broad cross-reactivity, may rely on regions of the native protein that are not exposed to the immune system or are exposed but are not naturally immunogenic, such as a cryptic epitope (*4*, *5*). Furthermore, intact proteins may contain additional epitopes that elicit undesired B-cell or T-cell responses. For example, full-length spike (S) glycoprotein-based vaccines of coronaviruses, including Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), have the potential to generate autoimmune responses resulting in liver damage of vaccinated animals and can exacerbate disease (*6–8*). Thus, while the full-length S protein is highly immunogenic and induces protection against SARS-CoV challenge in animal models (*9–11*), a SARS vaccine based on the full-length S protein may not be a safe and viable option for use in humans.

There are multiple issues that must be addressed for the successful generation of vaccines including off-target effects and limited efficacy (as determined by the ability of the vaccine to protect against pathogen challenge). Antibodies raised against a native protein often target epitopes that are not vital to the function or structure of the protein, or interact with epitopes that are prone to mutation. For example, when hemagglutinin (HA) or whole inactivated influenza virus is used as a vaccine the antibodies in the sera generally bind to the highly antigenic head unit, HA_1, which is also highly mutagenic. The effectiveness of these vaccines can vary across each flu season as a result of mutations to HA_1 (antigenic drift) (*12*). Few antibodies induced by whole HA protein or whole virus bind to the HA_2 stem unit, which is important for virus entry and is considerably more conserved than HA_1 (*13*, *14*). Recent studies have demonstrated that HA_2-specific antibodies can protect mice from the homotypic influenza virus strain and also cross-react with and neutralize several different subtypes of influenza virus (*15–19*). These antibodies bind to the HA_2 region of the fusion protein apparently preventing the conformational change in the stem, thereby blocking fusion of the viral envelope with host cell membranes. Thus, if one can elicit antibodies to functionally significant and/or highly conserved regions of a protein, the opportunity to provide a broadly protective vaccine is enhanced.

A potential way to specifically target functional and/or conserved regions in order to enhance the coverage/protection afforded by vaccines and to avoid vaccine-mediated toxicity is to present the minimal epitope required to produce a protective immune response (*20*, *21*). An epitope-based vaccine consists of a small, well-characterized protein epitope(s), which can be synthesized as a peptide(s) and appropriately modified to generate the desired immune response. The resulting immunogens can be used in active vaccination or to elicit therapeutic monoclonal antibodies against pre-selected epitopes from native proteins of pathogens. Synthetic peptides have been utilized for decades to design vaccines targeting a wide range of pathogens (*4*). While synthetic peptides are capable of eliciting antibodies that recognize the peptide itself, it is imperative that the antibodies generated by the peptide also recognize the correctly folded native proteins from which they were derived (*22*). The definition of a synthetic peptide vaccine, also referred to as an epitope-based peptide vaccine (*23*), is a vaccine in which a synthetic peptide immunogen(s) stimulates an immune response and elicits antibodies that protect against challenge by the pathogen.

Synthetic peptide vaccines have many advantages over traditional vaccines, particularly in regard to safety and ease of production (*21*, *24*, *25*). Although in traditional live attenuated vaccines the likelihood of viral reversion to virulence or incomplete inactivation of the virulent pathogen to make a killed vaccine is low (*26*, *27*), there is no risk of either process associated with peptide-based vaccines. Furthermore, advances in peptide synthesis technology, ease of introducing peptide modifications to control peptide conformation and stability and the available conjugation and cross-linking chemistry to attach peptides to carrier proteins make synthetic peptide vaccines attractive candidates for vaccine development. Moreover, the increasing availability of three-dimensional crystal structures of microbial protein targets, progress in deciphering key features of antibody-antigen interactions and advances in techniques to study binding processes have significantly eased the development of peptide-based vaccines. A major advantage of synthetic peptide immunogens is that specific epitopes can be synthesized and presented to the immune system, especially epitopes in the native protein that are not strongly immunogenic or whose immunogenicity is masked (*5*). Additionally, multiple epitopes from a single protein or from multiple protein targets can be designed into one synthetic peptide immunogen (*24*). This feature of synthetic peptide-based immunogens provides the potential for more diverse coverage of targets within a single pathogen, protection against antigenically diverse strains of a single pathogen or even immunization against more than one pathogen by means of a single peptide vaccine.

The Hodges laboratory has been very successful in developing synthetic peptide immunogens to raise antibodies against native proteins of pathogens. Kao et al. (*10*) developed a consensus sequence vaccine to target the receptor-binding domain of the type IV pilus of *Pseudomonas aeruginosa*. This study was followed by comparative analysis of antibodies raised to a synthetic peptide representing the receptor binding domain of a pilin protein (residues 128-144) of *P. aeruginosa* versus antibodies raised against the whole pilin protein monomer (residues 29-144) (*28*). The titers against the native pilin from sera of animals that were immunized with the synthetic peptide-conjugate were higher than those of the animals immunized with the pilin protein. Furthermore, the majority of antibodies raised against the pilin protein were not specific for the receptor-binding domain (*28*). Tripet et al. (*29*) reported a peptide immunogen with an epitope in the stem unit of the S protein of SARS-CoV. This synthetic peptide immunogen raised high titer, conformation-specific antibodies that were able to bind the S protein on the cell surface and neutralize the SARS-CoV (*29*).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

A last case in point on the utility of synthetic peptide vaccines is Pfizer's animal health drug, Improvest (referred to as Improvac outside of the US), a synthetic analog of gonadotropin releasing hormone (GnH) indicated for the prevention of boar taint in the pork industry (*30*, *31*). Rather than physically castrating male pigs, Improvest can be administered to generate neutralizing antibodies to natural GnH thereby preventing normal male maturation thus, preventing boar taint. This method is considered much more humane and sanitary compared to traditional methods of physical castration.

Although there are no synthetic peptide vaccines currently on the market for the treatment or prevention of human disease, several peptide vaccines are under investigation in clinical trials for vaccination against infectious agents and cancer. Much of the renewed interest in synthetic peptides as drugs is a result of significant advances in drug delivery and the ability to readily impart metabolic stability (*24*). Furthermore, the ability to induce a specific immune response through the design and inclusion of selected minimal epitopes allows for a level of control over the exact epitopes that are presented to the immune system, which was not previously achieved with large biologics.

It is clear that with the number of clinical trails involving peptide-based vaccines and the successful entrance of a synthetic peptide vaccine into the animal health market, we will soon witness the emergence of synthetic peptide vaccines in the human health market.

Constructing Synthetic Peptide Immunogens

Modifications To Enhance Immunogenicity and Impart Structure to Synthetic Peptides

Conformational preferences of peptides in solution can be correlated with the secondary structure present in the protein from which the peptides are derived (*32*, *33*). Anti-peptide antibodies recognize specific protein conformations (*34*, *35*) including denatured conformations (*36*). Potential drawbacks of synthetic peptide immunogens that may generate antibodies that have a weak affinity for the native protein and therefore confer only weak protection, include a lack of the specific conformation found in intact proteins and poor immunogenicity (*24*, *37*). Poor immunogenicity can be circumvented by conjugation to a carrier protein, administration of adjuvants or sequence modification. A lack of specific conformation can be addressed by grafting peptide epitopes into structures of similar conformation to the native protein, using a templated approach as described in this manuscript or by otherwise introducing conformational restrictions into the peptide (*28*, *38*, *39*). Since peptides alone tend to be poorly immunogenic, they are commonly conjugated to a carrier protein to enhance the immune response (*4*, *40*). This approach relies on the ability of the carrier protein to activate the immune response through the presentation of multiple T-cell epitopes. Common carrier proteins include keyhole limpet haemocyanin (KLH), tetanus toxoid, diphtheria toxoid, ovalbumin and serum albumins. Disadvantages of carrier protein conjugation methodology include a low level of control over conjugation reactions, poorly defined conjugates, epitope suppression by the carrier-protein and the potential for the generation of antibodies that cross react with the functional groups resulting from coupling chemistry (*20*, *41*, *43*). Nevertheless, there are a number of promising vaccine candidates in which the antigen is conjugated to a carrier protein. These potential vaccines target infectious disease agents such as influenza (*44*) and *E. coli* (*45*) as well as Alzheimer's disease (*46*) and cancer (*24*, *47*).

The use of defined T-cell epitopes allows one to avoid troublesome conjugation chemistry and epitope suppression or undesirable physicochemical characteristics mediated by carrier-proteins (*41*). In the early 1990s, Partidos et al. (*48*) reported a chimeric peptide comprised of a single B-cell epitope and a mouse MHC promiscuous T-cell epitope from the measles virus fusion protein that was capable of eliciting the desired immune response in mice. Kaumaya and co-workers have identified multiple chimeric peptides that target exogenous (e.g. virus) and endogenous epitopes in humans (*49–52*). In a recent phase I clinical trial, Kaumaya et al. (*53*) showed that a combination vaccine comprised of two synthetic peptides, each bearing a single T-cell epitope linked directly to a B-cell epitope of human epidermal growth factor receptor (HER2), was sufficient to inhibit the HER2 cancer pathway in patients who present with metastatic or recurrent tumors. Since the T-cell epitopes required for T-cell activation are short peptides, typically around 12-20 residues, their contribution to the physicochemical characteristics of the full length immunogen is expected to be dramatically reduced compared to typical carrier proteins which range from 60 kDa (Albumin carriers) to approximately 400-800 kDa for the KLH carrier (multimers consisting of approximately 50 kDa functional units) (*54*).

Further modification of peptide immunogens, beyond the inclusion of appropriate T-cell epitopes, is typically required to produce the desired immune response, particularly with regard to the structure of the synthetic peptide. There are numerous approaches available to introduce conformational constraints into peptide immunogens including cross-linking of peptide residues using covalent hydrogen bond mimics (*38*), lactam bridges (*55*), cyclization via amino acid side-chains (*56*) and disulfide bond formation (*39*). Disulfide bridging is one of the most popular techniques to constrain peptides for the generation of conformation specific antibodies, even against short peptides. Leonetti et al. (*57*) reported a synthetic octadecapeptide analog of toxin α (residues 23-40) isolated from the African cobra *Naja nigricollis* (*57*). This peptide was cyclized through a disulfide bridge of cysteine residues 23 and 40 to mimic a beta turn found in the native peptide (*58*) and was shown to induce antibodies in mice that cross-reacted with toxin α. In contrast, antibodies raised against the linear peptide analog with the same sequence only weakly cross-reacted with the native protein. Linear peptides are generally less immunogenic than their structured counterparts (*46*, *59*, *60*). However, caution must be taken, as the conformation of the restricted peptide may not be identical to the folded protein. In fact the cross-linked peptide could be less immunogenic than the unrestricted peptide (*60*). The conformational constraint of peptide immunogens to better mimic a given protein region may result in increased affinity of the anti-peptide antibodies for the parent protein even over that of protein immunogens. We recently demonstrated the advantages of a synthetic peptide immunogen over a protein subunit immunogen

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

in the development of an anti-pilus vaccine for *Pseudomonas aeruginosa* (*28*). The synthetic peptide immunogen had the same overall structure found in the native pilin protein that is, a disulfide bridge and two β-turns. Sera from animals immunized with the synthetic peptide-conjugate exhibited higher anti-pilin titers compared to those of animals immunized with the pilin protein subunit.

Strategies To Stabilize the Conformation of α-Helical Peptide Epitopes

Nearly 6% of proteins in the Protein Data Bank contain α-helical coiled-coil motifs (*61*), of which more than 90% show dimeric or trimeric interactions. Because coiled-coils readily oligomerize, they often function as part of larger protein complexes in a variety of important cellular processes (*62*). The coiled-coil motif has recently attracted attention as a promising drug target for the inhibition of viral membrane fusion and entry into host cells by viruses such as HIV (*63*), influenza (*18*, *64*) and SARS-CoV (*29*). Since α-helical coiled-coils represent important targets for the production of diagnostic, preventive and therapeutic antibodies (*65*), establishing technologies to present a stabilized α-helical antigen is of critical importance for the development of synthetic peptide-based vaccines. Coiled-coils possess a recurrent sequence periodicity comprised of heptad repeats, denoted $[abcdefg]_n$, where positions *a* and *d* are generally occupied by hydrophobic amino acids that are responsible for the formation and stability of secondary, tertiary and quaternary structures of coiled-coils (*66–70*).

Since synthetic peptides comprising α-helical regions tend to be unstructured in solution (*70*), it is important to introduce or impart structure to the peptide in order to maintain the neutralizing/protective capacity of antibodies to the epitopes in the native protein. There are several methods available to stabilize α-helical structures or restrict peptide epitope conformations assumed from the cognate sequence of the protein including 1) chemistry to introduce conformational constraint(s) 2) fusion to scaffolds, 3) sequence transplantation and 4) peptide templating.

Chemical modification and cross-linking can be employed to constrain the peptide epitope into a more rigid conformation and reduce its flexibility. Disulfide bond formation is commonly used to crosslink peptide antigens to various scaffolds in order to promote interactions between helical segments or to stabilize the desired conformation through intermolecular bridging. For example, Louis et al. (*71*) demonstrated inhibition of HIV-1 Env-mediated membrane fusion by truncated constructs of the glycoprotein 41 (gp41) fusion protein. The constructs comprised the internal helical region of gp41 and upon oxidation, intermolecular disulfide bridging formed a trimeric coiled-coil that elicited antibodies that were also capable of disrupting HIV fusion (*71*). Kaumaya and co-workers used a combination of scaffold constraint and peptide templating to develop peptide immunogens mimicking the human T-cell leukemia virus (HTLV-1) glycoprotein 21 (gp21) (*51*). Peptides representing B-cell epitopes were linked to a β-sheet scaffold in order to bring three polypeptide chains into the appropriate proximity to form a trimeric coiled-coil that was further stabilized by substitution of the *a* and *d* positions with leucine. The antibody elicited by this constrained trimeric

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

α-helical peptide immunogen recognized the native HTLV-1 fusion protein gp21 and reduced cell fusion induced by HTLV-1 gp21 (*51*).

The fusion or extension of α-helical epitopes with coiled-coil domains using either chemical or molecular biological techniques has been shown to stabilize α-helices as a result of downstream helical induction (*5*, *72*). The leucine zipper trimerization domain of the yeast transcription factor GCN4, has been linked to multiple peptide epitopes including the M protein of Streptococcus (*5*) and the eM2 of influenza (*73*). In these cases, extension of the peptide epitope with GCN4 resulted in immunogens that elicited antibodies that recognized the native protein. Nanoparticles based on self-assembling coiled-coil peptides comprising various oligomerization domains that mimic viral capsids also provide a potential scaffold for the systematic presentation of multiple peptide epitopes (*74*). This specific presentation of multiple B-cell epitopes was used to stabilize and present a trimeric coiled-coil comprising the C terminal heptad repeat (HRC) region of the fusion protein of SARS-CoV in a peptide nanoparticle (*75*). Antisera collected from mice immunized with the peptide nanoparticles neutralized virus infection *in vitro*.

A number of protein scaffolds are available for the transplantation and subsequent presentation of epitope sequences comprising the antigenic regions of various immunogens (*76*). Computationally aided design of 'epitope scaffold' immunogens has been successfully employed to elicit antibodies that recognize native proteins (*77*, *78*). The generation of such stabilized epitopes relies on the identification (matching) of an appropriate protein scaffold to present the side-chain residues responsible for generating an immune response. After "matching" the protein scaffold, the epitope is transplanted into the scaffold sequence. Mutagenesis is used to optimize the physicochemical and biological properties of the final immunogen. Epitope scaffolds from HIV-1 gp41, recognized by the broadly cross-reactive antibodies 2F5 (*78*) and 4E10 (*77*) and RSV, recognized by the neutralizing antibody motavizumab (*79*) were successfully generated by this strategy and they elicited high affinity antibodies.

The *de novo* design of stabilized helical proteins is also a successful strategy to develop immunogens to conformation-specific epitopes (*80–82*). One particular motif, the coiled-coil stem loop, an anti-parallel coiled-coil connected by an intervening loop, was shown to be an effective and compact scaffold for the presentation of epitopes as either loops or helices (*83*).

The fourth method for generating conformation-specific antibodies that recognize α-helices in proteins is the use of a novel two-stranded α-helical coiled-coil template developed by our group and others (*41*, *84*). Our template consists of a parallel, two-stranded, α-helical coiled-coil structure designed to maintain maximum stability through the substitution of Ile and Leu at heptad repeat $[abcdefg]_n$ positions *a* and *d*, respectively, to form the hydrophobic core of the coiled-coil. An interchain disulfide bridge further stabilizes the α-helical coiled-coil template. The surface-exposed residues from the helical sequence of interest are inserted into the template at positions *b, c, e, f* and *g*. Therefore, a minimum of five out of seven residues are unique to the α-helical sequence of interest. The two-stranded template is used to generate polyclonal antibodies, which are specific not only to the sequence of interest but also for its α-helical conformation. This approach allows for the facile generation of specific

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

conformation-dependent antibodies targeted against α-helical regions of native proteins. We have successfully utilized this technology in a large number of cases including the generation of antibodies that exhibited conformational-specificity for native GCN4 (*84*) and myotonic dystrophy protein kinase (*85*). More recently, we demonstrated the validity of this novel templated peptide approach to elicit neutralizing antibodies to conformation-stabilized α-helices in a class 1 viral fusion protein, SARS coronavirus spike (S) glycoprotein. We generated antibodies that specifically recognized the C-terminal heptad repeat HRC in the stem of the SARS-CoV S glycoprotein, and neutralized the infectivity of SARS-CoV *in vitro* (*29*).

The next sections detail our work on the presentation and stabilization of epitopes from the C-terminal heptad repeat coiled-coil of SARS-CoV S protein using our coiled-coil template. We will focus the discussion on our understanding of how the conformation of coiled-coil epitope peptides relates to immunogenicity (ability to elicit α-helical conformation-specific antibody) and antigenicity (ability to bind α-helical conformation-specific antibody).

Developing Alpha Helical Conformation-Specific Antibodies against Spike (S) Glycoprotein of Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)

SARS-CoV S Protein and Virus Entry

SARS-CoV is an enveloped positive-strand RNA virus that was responsible for the global outbreak of severe acute respiratory syndrome in 2003 and infected over 8,000 people with a fatality rate of approximately 10%. Like other enveloped viruses, SARS-CoV infects host cells through fusion of the viral envelope with the host cell membrane. Membrane fusion is mediated by the spike (S) protein, a class I viral fusion protein. As with most other class I viral fusion proteins, the S protein is comprised of two domains, an N-terminal receptor-binding domain (S1) and a C-terminal membrane fusion domain (S2). The S2 domain includes two conserved heptad repeat regions denoted HRN (heptad repeat N-terminal) and HRC (heptad repeat C-terminal) which form a six-helix bundle core in the postfusion conformation of S protein. The inner core is comprised of a coiled-coil trimer of HRN, which is flanked by three HRC helices arranged antiparallel and oblique to the HRN helices, ultimately forming the six-helix bundle (*86–88*).

Following receptor binding, enveloped viruses enter host cells by either direct fusion with the host cell plasma membrane (e.g. HIV) or through fusion with endosomal membranes (e.g. influenza) (*89*, *90*). SARS-CoV has the potential to infect host cells via either pathway depending upon the proteolytic cleavage between S1 and S2, pH and receptor binding (*91–95*)

Regardless of the pathway of infection, cleavage of S protein is a critical step for virus entry into cells. A programmed series of conformational changes from a prefusion to a postfusion state allows fusion of the viral envelope with the host membrane to release viral nucleocapsids into the host cells. Structural analysis of the HRC region (residues 1151-1185) suggests that in the prefusion state the HRC helices form a coiled-coil trimer (*88*). This same region interacts with HRN to form a six-helix bundle (*88*). The crystal structure of the postfusion state (six-helix bundle) shows residues 1160–1177 in HRC form a 5-turn α-helix with extended regions at the N- (residues 1150–1159) and C-termini (residues 1178–1184) (*86*). The N- and C- terminal residues of HRC change conformation from α-helical in the prefusion state to an extended conformation when bound to HRN in postfusion state. The intermediate fusion state of HRC is proposed to be an un-ordered monomer that exists in dynamic equilibrium with a coiled-coil trimer (*96*) prior to collapsing into the six-helix bundle comprised of the HRC and HRN regions of the S protein in the postfusion state. The S protein of SARS-CoV is not only vital for virus entry but is also important for cell-cell transmission (*92*) and immune recognition and response by the host (*97*) making it an important target for the development of SARS vaccines and therapeutics.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

HRC as a Target Epitope in the S Protein of SARS-CoV

We previously reported that sera containing antibodies to either HRC or HRN templated peptides bound to S protein in ELISA but only antibodies to HRC bound to the S protein expressed on the surface of Chinese hamster ovary (CHO) cells (*29*). Furthermore, only antibodies to the HRC peptide inhibited virus entry in an *in vitro* infectivity assay. Therefore, in the present study, the HRC region was targeted for the design of neutralizing antibodies against the S protein. It is reasonable to assume that the large conformational change between the prefusion state (three-stranded coiled-coil) and the postfusion state (six-helix bundle) requires an intermediate state with increased flexibility. This assumption is supported by recent NMR and biophysical studies (*96*, *98*). According to a proposed model of SARS-CoV entry (*98*), transformation from the prefusion state to the postfusion state proceeds through an unordered fusion-intermediate state. Binding of antibodies to the HRC region in the prefusion state could have at least two possible effects on its interaction with HRN: first, binding could stabilize the α-helical conformation, especially the regions composed of residues 1150-1160 and 1176-1185 in HRC, which in turn would reduce the ability of the N- and/or C-terminal regions of HRC to readily change conformation from α-helical to extended upon interacting with HRN; second, if the monomeric form of HRC in the fusion-intermediate state is the interacting species with HRN, then antibody that stabilizes the oligomeric structure would shift the monomer–trimer equilibrium in favor of the trimer and at some point diminish any monomer available to interact with HRN. Epitopes from the HRN and HRC coiled-coil domains were previously grafted into a two-stranded α-helical coiled-coil template and conjugated to a carrier protein as immunogens (*29*). These conjugates were highly immunogenic and raised high titer antibody responses in immunized rabbits. Two immunogens derived from the HRC region bound to native S protein (on virions or expressed on the cell surface) and inhibited SARS-CoV entry into cells. These results demonstrated that the HRC region is accessible to antibody binding and is vital to membrane fusion and the SARS-CoV entry process. Interestingly, the two immunogens to the HRC region were the same length but differed by a seven-residue shift in the sequence. This sequence shift has a dramatic effect on the desired properties of the antibodies showing the importance of the proper choice of sequence of the peptide immunogen (*29*).

Developing a Peptide-Based Vaccine for the SARS-CoV

In order to develop an efficacious vaccine, we examined the effects of stability, oligomeric state and flexibility of coiled-coil HRC peptide epitopes on immunogenicity and antigenicity. To gain insight into antibody-antigen interactions we addressed the following general questions: (1) How does the conformation of an antibody bound peptide compare to its conformation in solution and to that of the corresponding epitope in the native protein? (2) What are the implications of these conformational comparisons on the efficacy of the vaccine and on the mechanism by which anti-peptide antibodies are elicited? (3) What are the interactions between antibodies and their peptide antigens? (4) How do peptide-antibody interactions compare with protein-antibody interactions? We have analyzed the structures of our synthetic peptide immunogens, which correspond to the HRC region of the SARS-CoV S protein, in solution by biophysical methods and their ability to elicit conformation-specific antibodies, as well as the binding properties and viral neutralizing activity of antibodies elicited by these coiled-coil peptide immunogens. These studies provided insight into the structural features of peptide based epitopes that affect immunogenicity, cross-reactivity and specificity; the conformational relationship between peptide immunogen and the same region in native protein; and the development of a strategy to design a stable α-helical peptide vaccine that can elicit antibodies that recognize the native S protein in its prefusion conformation.

Design and Synthesis of Conformation-Specific Synthetic Peptide Immunogens To Elicit Antibodies That Recognize SARS-CoV S Protein

Many studies suggest that epitopes of native proteins are typically more complex than single, linear peptide moieties (*99*, *100*). During membrane fusion and viral entry, the functional unit of S2 is a trimeric parallel coiled-coil complex and not a monomer, and the trimeric S2 complex undergoes conformational changes during viral membrane fusion. Therefore, we speculate that a conformation-constrained peptide consisting of two or more HRC sequences locked in an α-helical coiled-coil may represent the conformation of the HRC epitopes recognized by neutralizing antibodies specific for the HRC region. Thus, it is of interest to investigate how the HRC epitope behaves as an immunogen in the monomer, dimer and trimer formats. We designed and synthesized three synthetic peptide immunogens containing the HRC epitope: one-stranded HRC, templated two-stranded HRC and peptide scaffold-three-stranded HRC. These three HRC peptide immunogens were each conjugated to KLH (Figure 1) in order to elicit anti-peptide antibodies *in vivo*. The same peptides were also coupled to bovine serum albumin (BSA) in order to study the binding properties of the three antibodies by ELISA.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

First, to examine the effect of the oligomeric state and the stability of the coiled-coil on immunogenicity, we conjugated a one-stranded HRC peptide to maleimide-activated carrier proteins, KLH or BSA using an N-terminal cysteine on the peptide to form a thioether. Second, we conjugated a disulfide-bridged templated two-stranded HRC peptide onto KLH through a benzoylbenzoyl moiety (BB), as previously described (*29*). As shown in Figure 2 and 3, a *de novo* designed template has been developed for generating conformation-specific antibodies that recognize α-helices in native proteins (*29*, *84*). This template consisted of a parallel two-stranded α-helical coiled-coil structure designed for maximum stability through an Ile/Leu hydrophobic core (Ile at all *a* positions and Leu at all *d* positions of the heptad repeat, $[abcdefg]_n$ for coiled-coils) and an interchain disulfide bridge at N-terminal position *a*. Surface-exposed residues from the helical sequence of interest (positions *b*, *c*, *e*, *f* and *g*) were inserted into the template. The templated peptides were synthesized by solid-phase peptide synthesis, purified, assembled into disulfide-bridged two-stranded templated peptides, conjugated to a carrier protein (KLH) and used for immunization of rabbits. The general outline of the experimental procedures used to prepare the templated peptide-carrier protein conjugate for immunization is shown in Figure 4. One HRC epitope site (1154-1179) was incorporated into the coiled-coil template. The templated α-helical epitope encompasses nine helical turns per helical strand. The length of the coiled-coil templated peptide is 31 residues, 18 of the 31 residues occur in the surface exposed *b*, *c*, *e*, *f*, and *g* heptad positions (-NAS-VN-QKE-DR-NEV-KN-NEL) and are responsible for the generation of antibodies that recognize the native S protein. Two Arg residues were included at the C-terminus of each strand to enhance the solubility of the templated peptide. The Ala residues at the N-terminus (positions *b* and *c*) provided a small helix forming spacer prior to the N-terminal Cys. Alanine has the highest α-helical propensity of the 20 amino acids (*101*) and is minimally immunogenic due to its small size. Finally, the N-terminus of one strand was acetylated while the second strand was extended by an additional norleucine residue and a glycine residue. The norleucine/glycine residues act as a spacer between the coiled-coil immunogen and the site of conjugation to the carrier protein, while norleucine allows for easy quantitation of the peptide/carrier ratio after conjugation by amino acid analysis. Benzoylbenzoic acid (BB) was coupled to the N-terminus of the extended strand. BB is a very efficient photo-activated cross-linker of synthetic peptides to carrier proteins (*102–104*). Third, we conjugated a three-stranded HRC peptide where the three HRC strands were coupled to a peptide scaffold onto KLH through BB, as shown in Figure 5. Briefly, we synthesized the HRC peptide epitope consisting of residues 1150-1185 and added a Cys-Gly-Gly linker at the N-terminus for covalent attachment to a peptide scaffold to form a covalently linked trimer (peptide scaffold-three-stranded HRC). To prepare the peptide scaffold we synthesized the peptide, BB-nLGKGKGKGRR-amide. After cleavage and purification, three bromoacetyl groups were added to three ε-amino groups of the three Lys residues by reacting with the N-hydroxysuccinimide ester of bromoacetic acid. The peptide scaffold-three-stranded HRC peptide was then prepared by addition of the HRC peptide with an N-terminal cysteine (Figure 5) to the bromoacetylated peptide scaffold.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Table 1. Molar ellipticity of the peptide immunogens used in this study. The CD spectra were recorded with 30 µM equivalents of HRC peptide in each construct in 0.1 M KCl, 0.05 M K_2HPO_4 buffer, pH 7.2 (benign conditions) and with 50% TFE in benign buffer

Peptide	*$[\theta]_{222}$ benign (degrees cm^2 $dmol^{-1}$)*	*$[\theta]_{222}$ 50% TFE (degrees cm^2 $dmol^{-1}$)*
One-stranded HRC	-21371	-30040
Peptide scaffold-three-stranded HRC	-27054	-31116
Disulfide-bridged templated two-stranded HRC	-31249	-30578

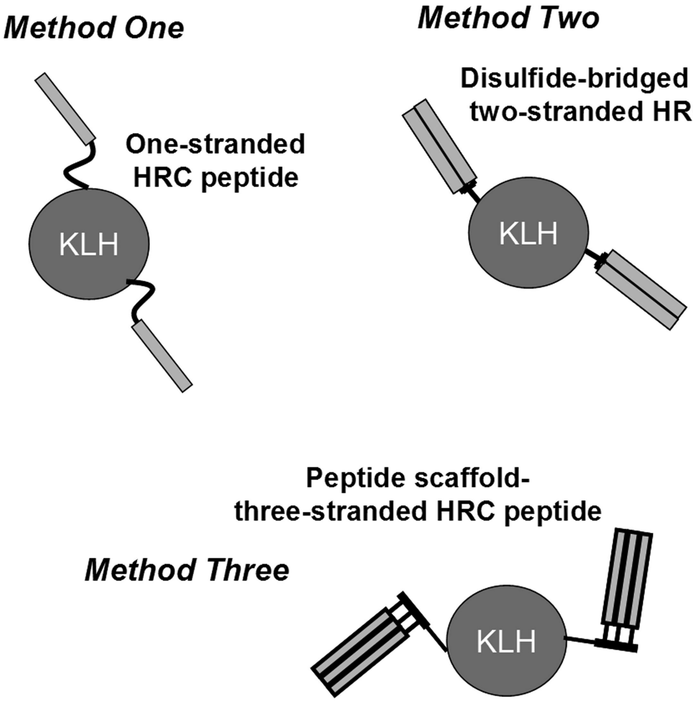

Method One
One-stranded HRC peptide
KLH
Method Two
Disulfide-bridged templated two-stranded HRC peptide
KLH
Peptide scaffold-three-stranded HRC peptide
Method Three
KLH

Figure 1. Design schematic of three different HRC immunogens used in this study. The one-stranded, templated two-stranded and peptide scaffold-three-stranded immunogens were covalently linked to keyhole limpet haemocyanin (KLH) as the carrier protein. This is a design schematic; the actual ratio of peptide to KLH as determined by amino acid analysis averages 4:1. We generally aim for a ratio of approximately 5:1 peptide:carrier protein.

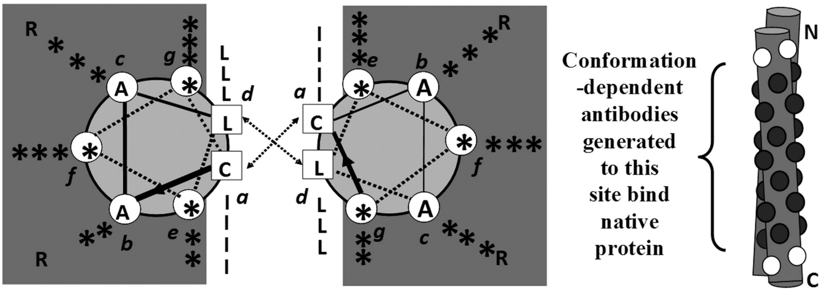
Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

abcdefgabcdefgabcdefgabcdefgabc

BB-nLG-CAAL*I**L***I**L***I**L***IRR-amide**
|
Ac-CAAL*I**L***I**L***I**L***IRR-amide**

The amino acid residues exposed on the surface (*) of the native virus α-helix are engineered into our highly stabilized synthetic peptide template


R
*
c
g
L
L
L
d
A
f
a
C
L
b
e
R
N
Conformation -dependent antibodies generated to this site bind native protein
C

gabcdefgabcdefgabcdefgabcdefgabcdef

Native Sequence HRC (1150-1185) DISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL

One-stranded HRC Immunogen CGG-IAAI**NAS**V**VN**I**QKE**I**DR**L**NEV**A**KN**L**NES**L-amide

Templated Two-Stranded HRC Immunogen
BB-nLG-CAAL**NAS**I**VN**L**QKE**I**DR**L**NEV**I**KN**L**NES**IRR-amide
|
Ac-CAAL**NAS**I**VN**L**QKE**I**DR**L**NEV**I**KN**L**NES**IRR-amide

Figure 2. Schematic of the two-stranded α-helical coiled-coil template. Top panel: the two-stranded α-helical coiled-coil template sequence. The 18 positions, which can be substituted with native S protein residues, are indicated with an asterisk (*). The letters [abcdefg]$_n$ denote the heptad repeat sequence where positions a and d are the non-polar residues responsible for the formation and stability of two-stranded α-helical coiled-coils. BB-nLG (Benzoylbenzoyl-norleucine-glycine) denotes the linker for conjugation to the carrier protein. Middle panel shows a cross-sectional view of the two-stranded coiled-coil template on the left looking into the page from the N-terminal of the sequence. A side view of the two-stranded coiled-coil is shown on the right. The bottom panel shows the 35-residue amino acid sequence of the native HRC (1150-1185). The a and d positions are underlined. The disulfide-bridged templated two-stranded HRC immunogen shows the native 18 HRC residues in bold at positions b, c, e, f and g that are inserted into the template. The one-stranded HRC immunogen contains the same sequence as shown in the templated two-stranded immunogen, except the N-terminal cysteine that forms the disulfide bridge at position a is replaced with Ile, and a CGG linker is used to couple the single strand to the carrier protein.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

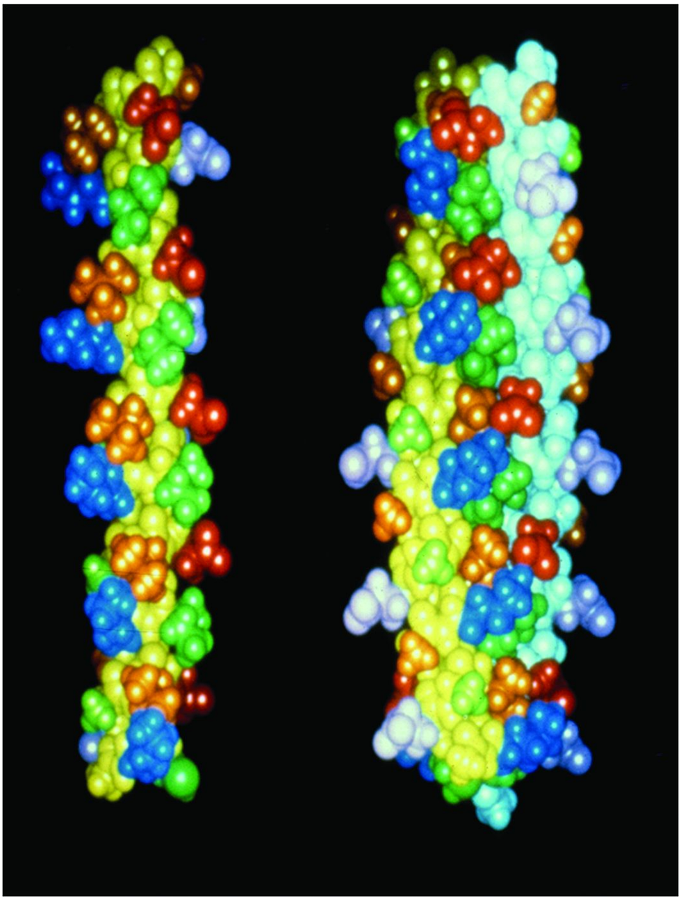


Figure 3. Space filling model of a 35-residue monomeric amphipathic α-helix (left panel) and a two-stranded α-helical coiled-coil (right panel). The green and brown residues shown on the monomeric α-helix (left panel) represent the non-polar residues at positions a and d of the heptad repeat [abcdefg]$_n$ that are responsible for the formation and stability of the coiled-coil. These residues form a continuous hydrophobic surface along the helix. These residues are buried in the hydrophobic core on formation of the two-stranded coiled-coil (right panel). In this model the polypeptide backbone of one α-helix is colored white and the other α-helix is colored yellow. The two α-helices are coiling about one another like a two-stranded rope. Interchain electrostatic attractions between lysine (blue) and glutamic acid residues (red) at position g and e' (i to i'+5) are observed in this model coiled-coil and cross over the hydrophobic interface between the two helices and further bury the hydrophobic core residues at positions a and d. The first high resolution structure of a parallel two-stranded α-helical coiled-coil shows that positions a and d are almost totally buried in the dimer (67). (see color insert)

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

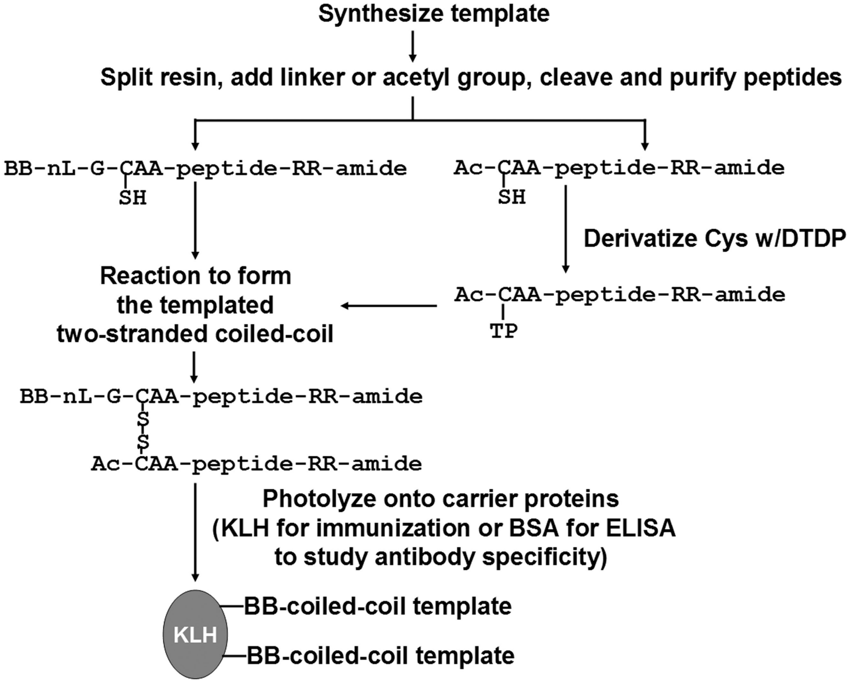

Synthesize template
Split resin, add linker or acetyl group, cleave and purify peptides
BB-nL-G-CAA-peptide-RR-amide
SH
Ac-CAA-peptide-RR-amide
SH
Derivatize Cys w/DTDP
Reaction to form the templated two-stranded coiled-coil
Ac-CAA-peptide-RR-amide
TP
BB-nL-G-CAA-peptide-RR-amide
S
S
Ac-CAA-peptide-RR-amide
Photolyze onto carrier proteins (KLH for immunization or BSA for ELISA to study antibody specificity)
KLH
BB-coiled-coil template
BB-coiled-coil template

Figure 4. The general outline of the experimental procedures used to prepare the templated two-stranded peptide-conjugates (KLH or BSA) for immunization or ELISA. BB, denotes the photoprobe, benzoylbenzoyl, used for covalent attachment of the peptide to the carrier protein (See Materials and Methods section for further details). DTDP is the reagent 2,2'-dithiodipyridine, TP refers to the thiopyridine group attached to the sulfur atom of cysteine.

CD Analysis of the Synthetic HRC Peptide Immunogens

Circular dichroism (CD) spectroscopy is a relatively simple technique used to estimate the extent of secondary structure in peptides (or proteins) averaged over the entire sequence. In Figures 6a, 6b and 6c, the CD spectra of three peptide immunogens exhibited characteristic double minima at 208 and 222 nm, typical of α-helical structure. As shown in Table 1, the molar ellipticities at 222 nm are -21371 for one-stranded HRC, -27054 for peptide scaffold-three-stranded HRC and -31249 for templated two-stranded HRC, respectively, in benign (non-denaturing) buffer. The theoretical molar ellipticity for a fully helical 31-residue peptide is −34070 (*105*). When the HRC peptide immunogens were analyzed in 50% trifluoroethanol (TFE, a helix inducing solvent), the molar ellipticity, increased to -30040 for one-stranded HRC, to -31116 for peptide scaffold-three-stranded HRC. However, the ellipticity of the disulfide-bridged templated two-stranded HRC decreased due to the fact that the peptide was already fully folded in benign buffer (non-denaturing aqueous conditions). It

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

has been shown that when TFE dissociates, the two helices of a coiled-coil into single stranded helices the molar ellipticity ratio 222/208 nm changes. For single stranded α-helices in TFE the 222/208 nm ratio is less than 1. For coiled-coils in aqueous media the 222/208 nm ratio is greater than 1 (*69*, *106*). Thus, our data indicate that all three peptide immunogens can form α-helical structures under benign conditions. The templated two-stranded HRC conjugate possessed the highest content of α-helical structure and demonstrated a fully folded α-helical state. These three peptide immunogens were used to study the effect of α-helical content and oligomeric state on immunogenicity.

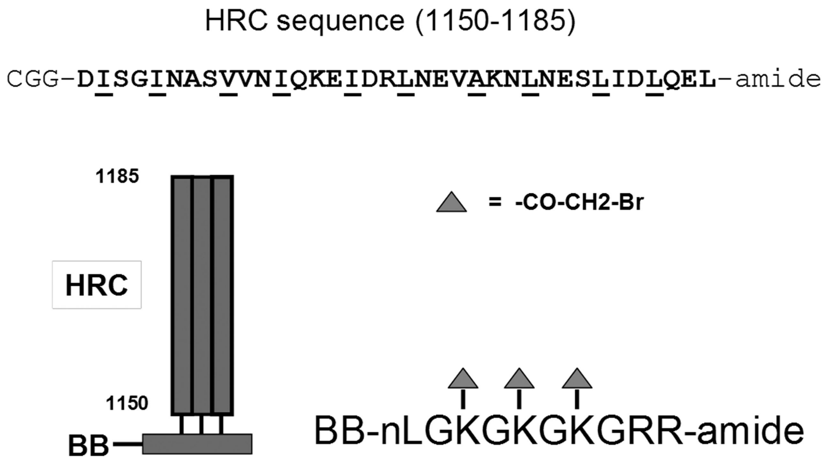


Figure 5. Schematic for the preparation of the peptide scaffold-three-stranded HRC immunogen showing the sequence of the scaffold used to anchor the three strands of HRC. Three bromoacetyl moieties on the peptide scaffold are reacted with the cysteine residue at the N-terminus of three HRC peptides. The BB moiety is used to attach the peptide scaffold-three-stranded HRC peptide to the carrier protein (either KLH to be used as an immunogen or BSA to screen for antipeptide antibodies).

Immunogenicity and Specificity of Antibodies Elicited by Three Synthetic HRC Peptide Immunogens

In order to evaluate and compare their immunogenicity, each peptide immunogen (one-stranded, two-stranded, and three-stranded peptides coupled to KLH) was administered to New Zealand White rabbits according to standard animal protocols (*29*). The initial injection of the peptide immunogen contained complete Freund's adjuvant while subsequent booster shots (3) consisted of peptide in incomplete Freund's adjuvant. After day 58, final sera were collected and antibodies (IgG) were purified by Protein G affinity chromatography. The binding properties of the antibodies elicited against the peptide immunogens were evaluated by enzyme-linked immunosorbent assays

(ELISAs). The wells of the ELISA plate were coated with the BSA-conjugated peptide immunogens. As shown in Figure 7, each of the antibodies bound to its corresponding peptide antigen demonstrating that each synthetic HRC peptide conjugate was immunogenic. Antibodies elicited against one-stranded, two-stranded, and three-stranded peptide immunogens bound similarly to peptide scaffold-three-stranded HRC peptide-BSA conjugate (Figure 7B). Both the antibody to templated two-stranded HRC immunogen and the antibody to the one-stranded HRC immunogen bound similarly to the one-stranded HRC-BSA conjugate while the antibody to the peptide scaffold-three-stranded HRC immunogen bound slightly weaker (Figure 7A). The antibody to the templated two-stranded HRC immunogen bound the two-stranded HRC peptide-BSA conjugate much stronger than the other two antibodies (Figure 7C). These data show that antibodies to these three peptide immunogens have different binding specificities related to their oligomeric state.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Ability of Antibodies to the One- and Two-Stranded Peptide Immunogens To Bind to SARS-CoV S Protein Expressed on the Surface of HEK293T Cells

Antibodies raised against the different HRC immunogens were examined for their ability to bind native trimeric SARS-CoV S protein displayed on the surface of HEK293T cells. Here, the S protein is assumed to be in its prefusion conformational state since it is not bound to its target receptor and the S1 and S2 domains are intact (*29*). HEK293T cells were grown to 70-80% of confluence then transfected with the pcDNA3.1-SARS-S Δ19 plasmid, which encodes for full length SARS-CoV S protein but lacks 19 C-terminal residues allowing for the S protein to be expressed and transported to the cell surface. The cells were incubated with antibodies to the HRC peptide immunogens followed by reaction with phycoerythrin (PE)-conjugated goat anti-rabbit antibody. The ability of antibodies to the one-stranded and disulfide-bridged templated two-stranded peptide immungens to bind the cell surface S protein was determined by fluorescence activated cell sorting (FACS, flow cytometry) and the results are presented as the percent increase in fluorescence intensity relative to mock transfected cells. As shown in Figure 8 and Table 2, the antibody to the templated two-stranded HRC immunogen bound to the S protein on the cell surface of HEK293T cells while little or no binding was observed for antibody to the one-stranded HRC immunogen. These results indicate that the conformation of the native S protein expressed on the surface of HEK293T cells is such that the HRC coiled-coil is clearly accessible to the antibody elicited by the templated two-stranded coiled-coil HRC peptide immunogen. Despite the observation that the antibody to the one-stranded HRC immunogen bound to the trimeric coiled-coil HRC peptide-BSA conjugate, it did not bind to the cell surface-exposed trimeric S protein on HEK293T cells. Our interpretation is that this antibody does not bind the HRC epitope in the prefusion conformation but the antibody elicited by the templated two-stranded HRC peptide immunogen does recognize and bind the prefusion state of the S protein.

Table 2. **Summary of Binding Properties and Inhibition of Virus Entry of HRC Antibodies. The one-stranded HRC, disulfide-bridged templated two-stranded HRC and peptide scaffold-three-stranded HRC were conjugated to KLH for immunization. The antibodies to these peptide immunogens were tested for binding to peptide scaffold-three-stranded HRC-BSA conjugate (Antigen 1) and the S protein expressed on the cell surface (Antigen 2) and for inhibition of virus entry.**

Antibody (Ab)	*Binding*		*Inhibition of Virus Entry*
	Antigen 1	*Antigen 2*	
Ab to one-stranded HRC peptide	+	-	-
Ab to disulfide-bridged templated two-stranded HRC	+	+	+
Ab to peptide scaffold-three-stranded HRC	+	-	-
Pre-immune IgG	-	-	-

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Ability of Three Different HRC Antibodies To Neutralize SARS-CoV Infectivity and Inhibit Cell-Cell Fusion Induced by S Protein

The ability of the different HRC antibodies to inhibit virus entry of SARS-CoV and prevent cell-cell fusion induced by S protein was assessed in a viral entry assay using a pseudotype virus as described in the methods section. SARS-CoV infectivity could not be neutralized by antibodies to one-stranded or peptide scaffold-three-stranded HRC immunogens nor by pre-immune rabbit sera (Figure 9). However, the antibody to the templated two-stranded HRC immunogen effectively neutralized the infectivity of SARS-CoV. Similarly, only this antibody could inhibit SARS-CoV induced cell-cell fusion (Figure 10 and Table 2). Based on the description of SARS-CoV cell entry pathways, virus entry in the viral neutralization assay used here is assumed to be through the endosomal pathway, in which the conformational change of the HRC epitope in the S protein only occurs in the endosome. Since, antibodies cannot penetrate the cell to bind the HRC epitope, the antibody to the templated two-stranded HRC immunogen most likely inhibited virus entry by interacting with the prefusion state of trimeric S protein of SARS-CoV (*29*).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

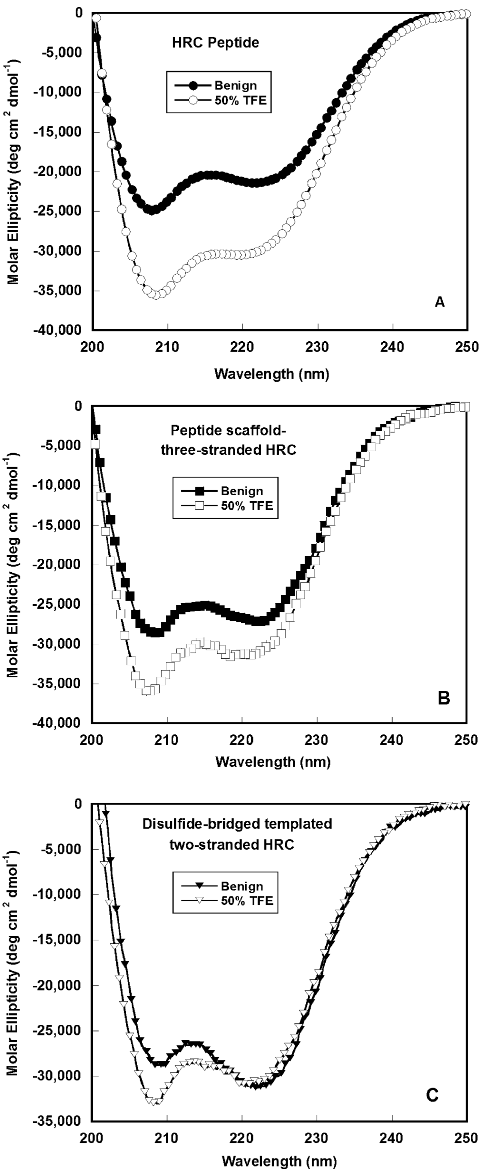


Figure 6. Far UV CD spectra of the SARS-CoV S immunogen peptides. Spectra were recorded in a 0.1 M KCl, 0.05 M K_2HPO_4 (benign) buffer, pH 7.2. The peptide concentrations were 30 μM for the one-stranded peptide (panel A), 10 μM for the peptide scaffold- three-stranded peptide (panel B) and 15 μM for the templated two-stranded peptide (panel C).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

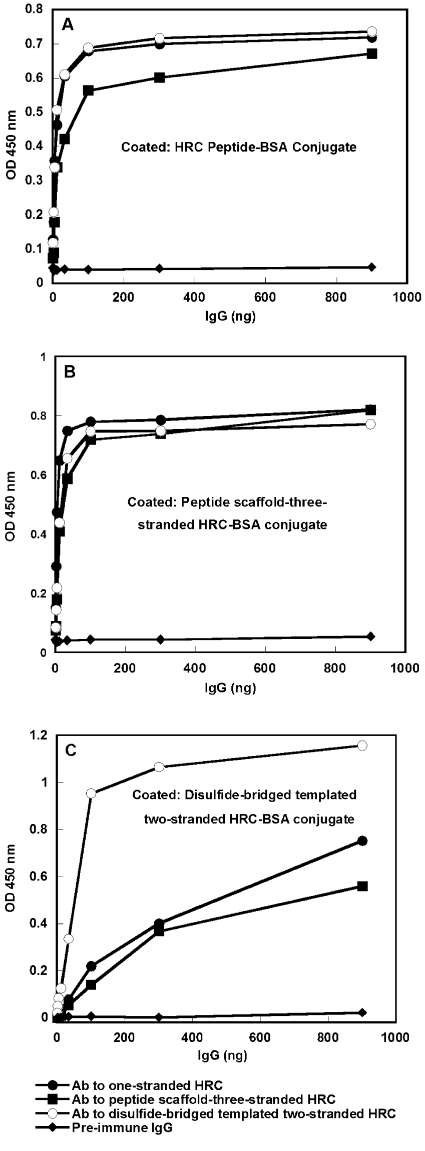


Figure 7. ELISA reactivity of anti-HRC antibodies with a panel of HRC synthetic peptides. Panel 7A. Coated HRC peptide denotes single stranded HRC (1151–1180) peptide conjugated to BSA. Panel 7B. Peptide scaffold-trimeric HRC (1150–1185) peptide conjugated to BSA. Panel 7C. Disulfide-bridged templated two-stranded HRC (1151–1180) peptide conjugated to BSA. Serial (threefold) dilutions of the antibodies were applied to the peptide (0.2 µg/well) and the amount of bound antibodies measured by an ELISA assay. The background was estimated by the amount of antibody bound to BSA and subtracted.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

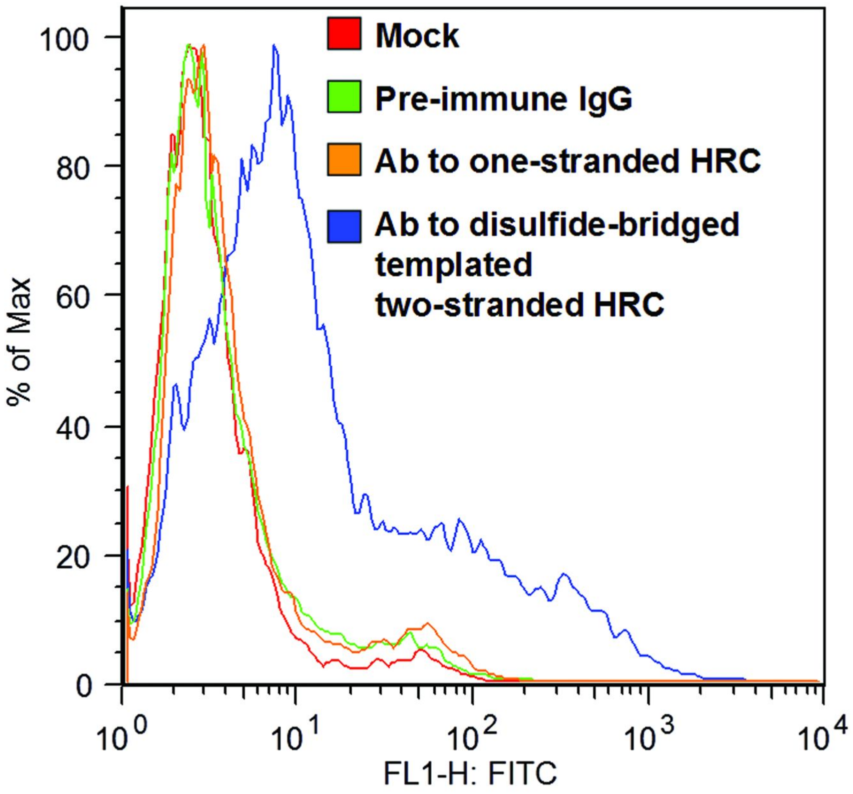


Figure 8. Binding of anti-HRC antibodies to SARS-CoV S glycoprotein expressed on the cell surface. The HEK-293T cells expressing SARS-SΔ19 protein were labeled with antibodies against either one-stranded or two-stranded HRC, followed with a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody. Cells were analyzed by flow cytometry. Red line: mock-transfected cells with antibody to templated two-stranded HRC; green line: HEK-293T cells expressing SARS-SΔ19 with pre-immune IgG; orange line: HEK-293T cells expressing SARS-SΔ19 with antibody against one-stranded HRC; blue line: HEK-293 cells expressing SARS-SΔ19 stained with antibody against templated two-stranded HRC. (see color insert)

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

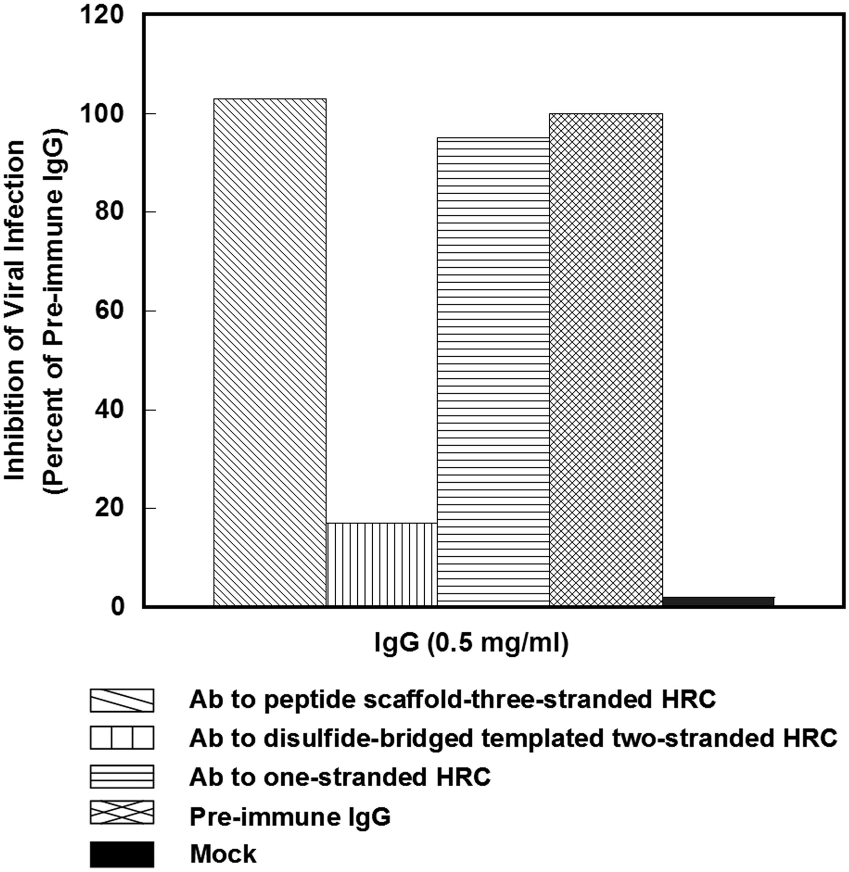


Figure 9. Antibody to templated two-stranded HRC significantly inhibited entry of SARS-CoV S pseudotyped retrovirus. Ten-fold serial dilutions of green fluorescent protein (GFP)-expressing retroviruses pseudotyped with SARS-SΔ19 glycoprotein were incubated for 30 min at 37 °C with different anti-HRC antibodies, and then the antibody-virus mixture was inoculated onto 293T cells expressing recombinant human angiotensin converting enzyme 2 (hACE2), the SARS-CoV receptor. After 24 h incubation, cells expressing GFP were enumerated to measure the efficiency of S-mediated virus entry. Infection by SARS-SΔ19 pseudotyped retrovirus in the presence of pre-immune IgG was set as 100%.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

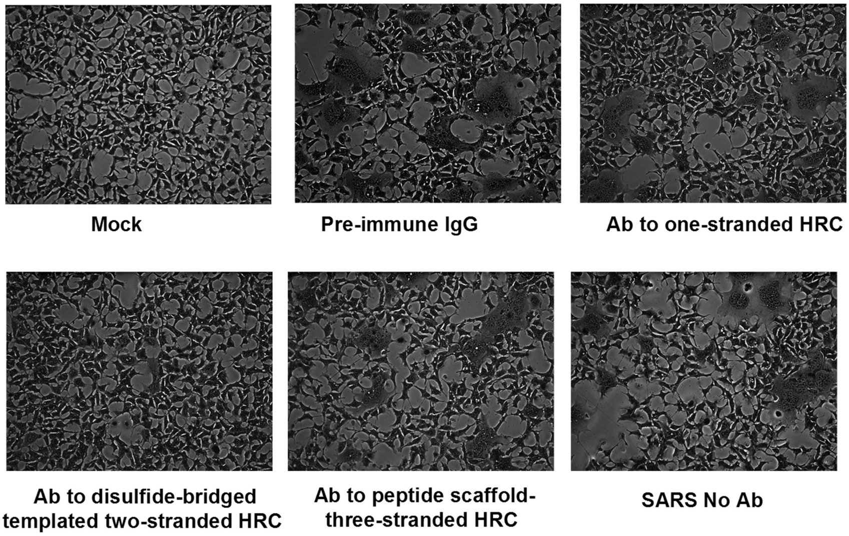

Mock
Pre-immune IgG
Ab to one-stranded HRC
Ab to disulfide-bridged templated two-stranded HRC
Ab to peptide scaffold-three-stranded HRC
SARS No Ab

Figure 10. Inhibition of SARS S protein-mediated syncytia formation by anti-HRC antibodies. HEK-293T cells expressing SARS-SΔ19 protein were incubated with trypsin to potentiate receptor-induced conformational changes in the S protein, and then overlaid onto a monolayer of 293/hACE2 cells at ratio of 1:3 in the presence of 1mg/mL of various anti-HRC antibodies. After 3 h incubation, cells were fixed with crystal violet containing fixative. Representative images from three random fields of cells incubated with different anti-HRC antibodies are shown.

Design of Three HRC Constructs To Mimic Different Conformational States of HRC

The above results raised the possibility that one-stranded, two-stranded and three-stranded HRC immunogens may elicit conformation-specific antibodies that target the HRC region of the S protein in different conformational states. Therefore, we designed three HRC constructs (Figure 11): 1) GCN4-HRC-GCN4 where both ends of the HRC peptide are stabilized by a modified GCN4 coiled-coil trimer. This construct did not bind to a HRN peptide, suggesting that HRC in this construct is stabilized in the trimeric prefusion state. 2) HRC-GCN4 where the C-terminus of the HRC peptide is stabilized by trimeric GCN4. This trimeric HRC construct bound to a HRN peptide, suggesting that the HRC peptide in this construct is in a more flexible state, similar to the putative fusion-intermediate states; 3) HRC in the HRC-HRN six-helix bundle which is presumed to be in a postfusion conformation. The oligomeric state and the ability of the constructs to bind the HRN peptide were confirmed by size exclusion chromatography (data not shown). In order to maximize stability, the GCN4 sequences were added directly in frame with the coiled-coil heptad repeat periodicity of the HRC peptide at the C-terminus for HRC-GCN4 and at the C- and N-terminus for GCN4-HRC-GCN4, as shown in Figure 11. The procedure to prepare HRC-HRN six-helix bundle and corresponding biophysical characterization was previously described (*88*, *100*).

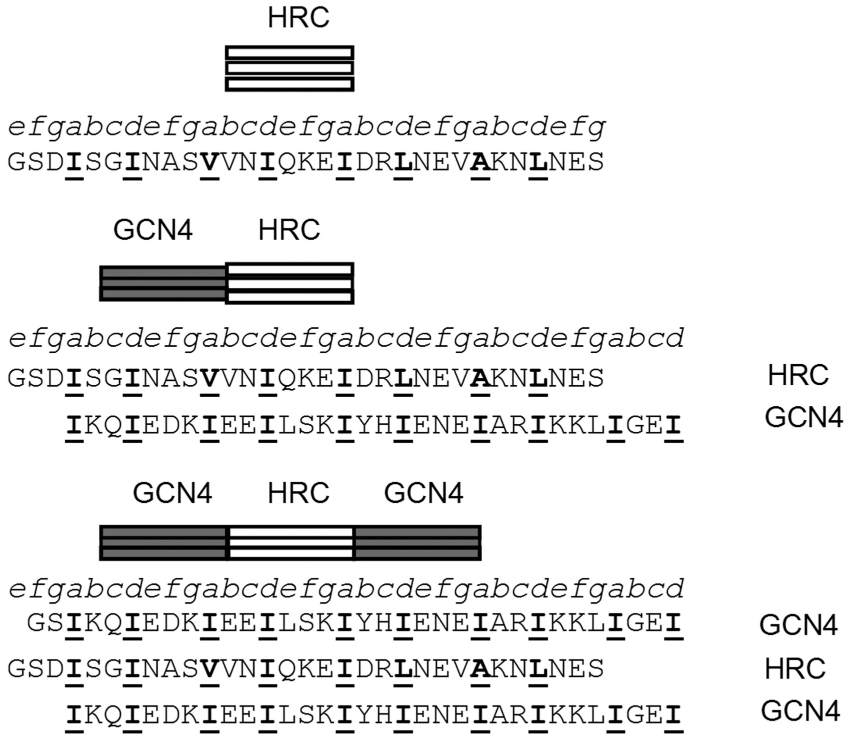

HRC
efgabcdefgabcdefgabcdefgabcdefg
GSDISGINASVVNIQKEIDRLNEVAKNLNES
GCN4
HRC
efgabcdefgabcdefgabcdefgabcdefgabcd
GSDISGINASVVNIQKEIDRLNEVAKNLNES
HRC
IKQIEDKIEEILSKIYHIENEIARIKKLIGEI
GCN4
GCN4
HRC
GCN4
efgabcdefgabcdefgabcdefgabcdefgabcd
GSIKQIEDKIEEILSKIYHIENEIARIKKLIGEI
GCN4
GSDISGINASVVNIQKEIDRLNEVAKNLNES
HRC
IKQIEDKIEEILSKIYHIENEIARIKKLIGEI
GCN4

Figure 11. Schematic of GCN4-HRC-GCN4 and HRC-GCN4 constructs with their corresponding sequences. The hydrophobic residues at positions a and d are bolded and underlined. The GCN4 mutant containing Ile residues at positions a and d forms a stable three-stranded coiled-coil.

CD Analysis of GCN4 Stabilized HRC Constructs and Synthetic HRC Peptides

As shown in Figure 12, both HRC-GCN4 and GCN4-HRC-GCN4 were fully folded α-helical coiled-coils in benign buffer. When the measurements were performed in 50% TFE, there was essentially no increase in the helicity, as measured by ellipticity at 222 nm, of either construct. This implies that these peptides attained their maximum helical content in PBS and that GCN4 at the C-terminus of HRC or at both the N- and C-termini increased and stabilized the trimeric α-helical structure of HRC.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

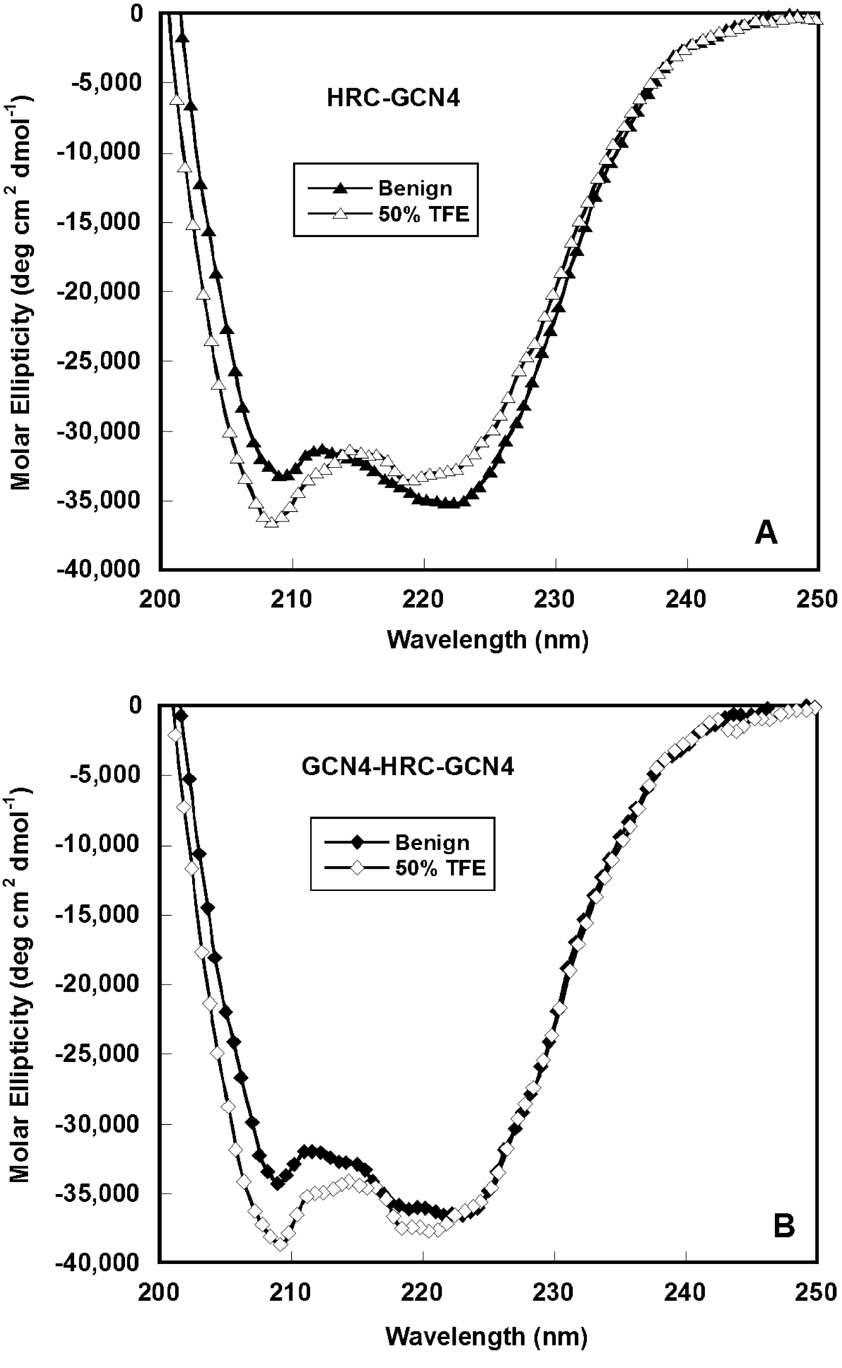


Figure 12. Far UV CD spectra of the GCN4-HRC-GCN4 and HRC-GCN4 constructs. Spectra were recorded in a 0.1 M KCl, 0.05 M K_2HPO_4 buffer, pH 7.2. Peptide concentrations were 30 μM equivalent of HRC peptide.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

To assess the stability of the HRC constructs, each peptide was thermally denatured and the change in structure from a folded α-helical state to an unfolded state was monitored using CD spectroscopy at 222 nm. The thermal denaturation profiles of the peptides are shown in Figure 13. Both GCN4-HRC-GCN4 and HRC-GCN4 are thermally stable, exhibiting a 20% decrease of α-helical structure at 85 °C compared to the fully folded state. These constructs were used in ELISA to mimic the prefusion conformation and the more flexible conformation of the proposed intermediate state of the HRC region of the S protein during the viral fusion process.

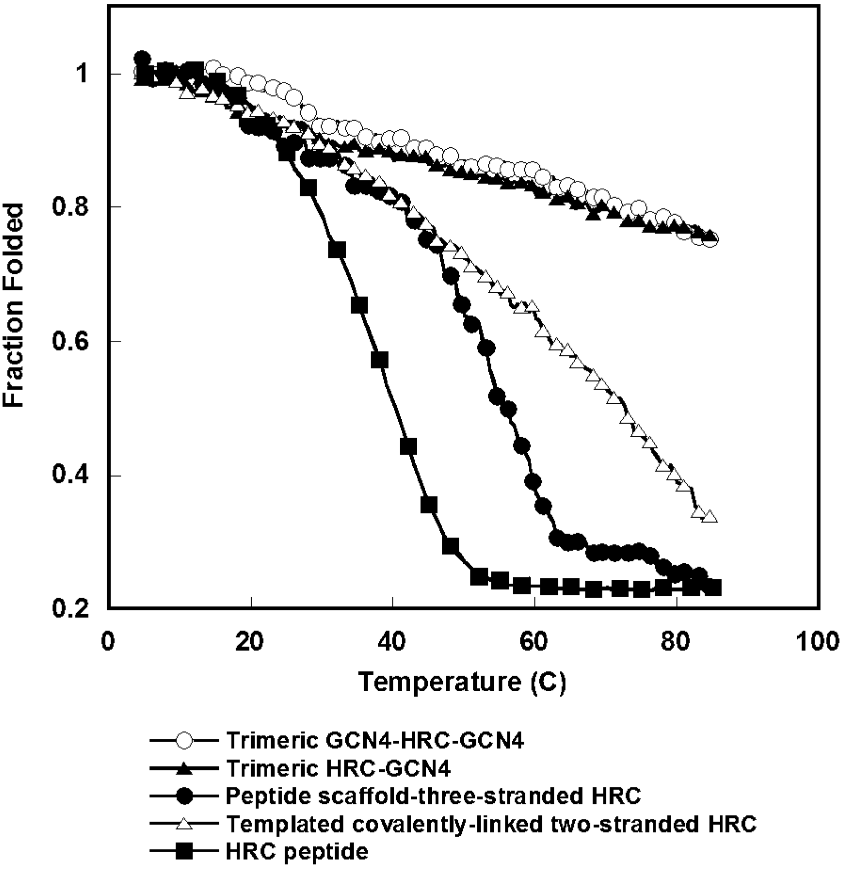


Figure 13. Temperature denaturation profiles of the three helical HRC peptides (one-stranded, templated two-stranded and peptide scaffold-three-stranded) and the two-GCN4 stabilized constructs. Denaturation was monitored by CD at 222 nm in a 0.1 M KCl, 0.05 M K_2HPO_4 buffer, pH 7.2. Peptide concentrations were 30 μM equivalent of HRC peptide.

The templated two-stranded HRC peptide also displayed a very stable thermal denaturation profile with a temperature denaturation midpoint of approximately 72 °C. It was significantly more stable than the peptide scaffold-three stranded HRC peptide which had a thermal denaturation mid point of 55° C. The one-stranded peptide (at 30 μM to favor oligomer formation) was the least stable peptide with a temperature denaturation midpoint of 38 °C (Figure 6 and Table 1).

Ability of Antibodies to Three HRC Peptide Immunogens To Bind Three Conformationally Defined Antigenic Peptides

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

The binding properties of the anti-HRC antibodies elicited against the three synthetic peptide immunogens (one-stranded, two-stranded, and three-stranded) were examined by ELISA. High binding plates (96-well) were coated with the three constructs of HRC (GCN4-HRC-GCN4, HRC-GCN4 and six-helix bundle; Figure 14). Despite the fact that each of the three antibodies bound similarly to its corresponding peptide-BSA conjugate (Figure 7) the reactivity of the three antibodies with the HRC constructs varied considerably. Though the antibody to the one-stranded HRC immunogen bound to all three constructs, it bound significantly stronger to the six-helix bundle construct compared to the other two HRC antibodies (elicited by templated two-stranded or peptide scaffold-three stranded HRC immunogens) as shown in Figure 14, **Panel C**. Thus, the antibody to the one-stranded HRC immunogen preferentially binds monomeric HRC as it is presented in the six-helix-bundle where each of three HRC strands is bound separately in each of the three grooves formed by the helices of the central HRN trimer. The helical content of the HRC peptide in the six-helix bundle is reduced due to the conformational change of N- and C- terminal residues from α-helical (prefusion) to an extended conformation (post fusion). The antibody to the peptide scaffold-three-stranded HRC immunogen recognized all three constructs but bound weaker to the GCN4-HRC-GCN4 and six-helix bundle constructs relative to the other HRC antibodies (Figure 14, **Panels A** and **C**). This suggests that this antibody preferentially binds HRC in its more flexible trimeric state as shown in Figure 14, **Panel B**. Interestingly, compared to the other antibodies, the antibody to the templated two-stranded HRC immunogen bound most strongly to HRC in its most stable trimeric state (GCN4-HRC-GCN4, Figure 14, **Panel A**), and bound most weakly to HRC in the six-helix bundle state (Figure 14, **Panel C**). These results help to explain why the only antibody that binds to the S protein on the cell surface (presumably in the trimeric prefusion conformation) and inhibits virus entry is the antibody to the templated two-stranded HRC immunogen. That is, the antibody to the templated two-stranded HRC immunogen can recognize the HRC epitope in the native S protein in its prefusion conformation and block the conformational change to inhibit virus entry.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

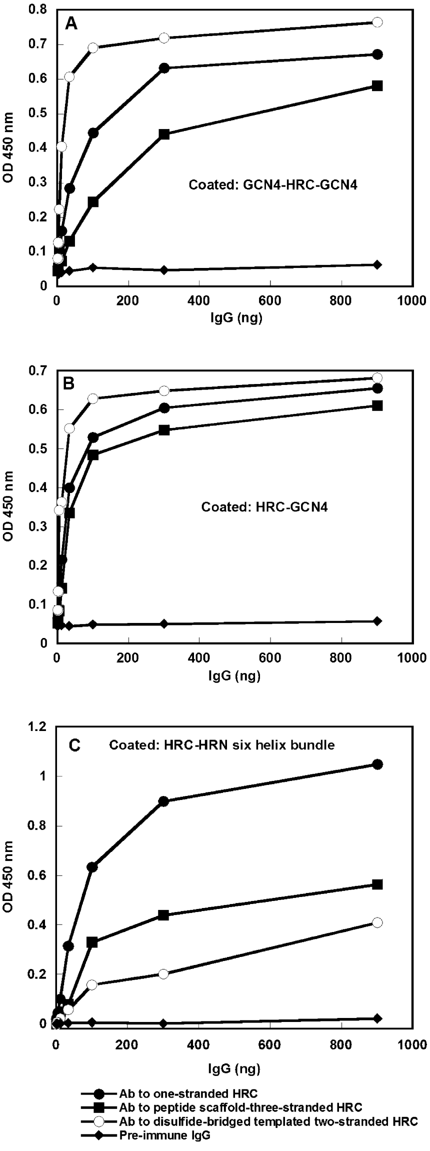


Figure 14. ELISA of anti-HRC antibodies against three different HRC constructs plated in ELISA wells. Panel A: Wells were coated with GCN4-HRC-GCN4 (a proposed mimic of the prefusion state of SARS-CoV S protein), Panel B: Wells were coated with HRC-GCN4 (a proposed mimic of the intermediate fusion state) and Panel C: Wells were coated with HRC-HRN complex (six-helix bundle; a mimic of the postfusion state). Serial (threefold) dilutions of the antibodies were applied to the peptide (0.2 µg/well) and the amount of bound antibodies measured by an ELISA assay. The background was estimated by the amount of antibody bound to BSA and subtracted.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Discussion

In order to generate antibodies that recognize a specific conformation in a native protein, it is necessary to develop suitable methods to constrain a peptide immunogen so that it mimics the same domain found in the native protein in terms of stability and flexibility. The present study focused on developing an efficient strategy to design a peptide immunogen to elicit α-helical conformation-specific antibodies that recognize the native protein. This work was conducted employing a single epitope from the C-terminal heptad repeat (HRC) region of the S protein of SARS-CoV (Figure 2).

Immunogenicity of the Synthetic HRC Peptide Immunogens

Antibodies raised to a peptide tend to bind the peptide with high affinity but have lower affinity for the native protein from which the peptide is derived (*36*). There are specific features that are important in the peptide immunogen to produce antibodies that bind the native protein. In addition to structural mimicry, the flexibility of the peptide immunogen should be optimized in order to elicit antibodies with the desired binding specificity (*38*, *58*, *107*, *108*).

Although the three HRC peptide-immunogens (one-stranded HRC-, templated two-stranded HRC- and peptide scaffold-three-stranded HRC-KLH conjugates) presented here contained the same HRC epitope and each immunogen elicited highly titer antibodies to its peptide antigen (peptide-BSA conjugates), the resulting antibodies demonstrated a wide range of affinities to the GCN4 constructs, BSA-conjugates and the native S protein and had different virus neutralizing activities.

The one-stranded HRC-KLH conjugate was not constrained nor stabilized as an α-helix. The antibody raised to this peptide did not demonstrate an explicit conformational specificity except that it did not bind the S protein expressed on the surface of HEK293T cells. Of the antibodies studied, the antibody to one-stranded HRC immunogen had the lowest affinity for the templated two-stranded HRC-BSA conjugate and the highest affinity for the postfusion six-helix bundle construct (Figure 7 and 14). Since, this antibody could not recognize the native S protein in its prefusion conformation, it was not able to inhibit virus entry.

The peptide scaffold-three-stranded HRC peptide immunogen demonstrated intermediate stability between the one-stranded HRC peptide and templated two-stranded HRC peptide in thermal denaturation studies (Figure 13). The antibody elicited by this peptide immunogen exhibited the weakest binding to the trimeric GCN4-HRC constructs (Figure 14A and B) as well as to the one-stranded and templated two-stranded peptide-BSA conjugates (Figure 7A and C). This antibody also had relatively weak binding to the postfusion conformation of HRC (monomeric HRC in each of the three grooves of the six-helix bundle, Figure 14C).

The templated two-stranded HRC peptide had the highest helical content and was the most thermally stable peptide immunogen of the three peptide immunogens examined here (Figure 6C and 13). Importantly, the antibody elicited against this peptide was the only antibody capable of binding the prefusion

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

state of the native S protein, preventing virus entry and inhibiting S protein mediated cell-cell fusion. This antibody exhibited the strongest binding to the two GCN4 constructs (HRC-GCN4 and GCN4-HRC-GCN4, Figure 14) and had an affinity for the one-stranded and peptide scaffold-three-stranded peptide-BSA conjugates that was similar to the antibodies to the one-stranded and peptide scaffold-three-stranded HRC peptide immunogens, respectively (Figure 7). The antibody to the templated two-stranded HRC immunogen also had the highest affinity for the templated two-stranded HRC peptide-BSA conjugate (Figure 7C) and the weakest affinity for the postfusion conformation of HRC (six-helix bundle, Figure 14C).

Perhaps as a result of the greater flexibility of the one-stranded and peptide scaffold-three-stranded peptides, the antibodies elicited against these peptide immunogens likely recognize several conformations of the HRC region, only a small percentage of which match that of the native S protein in its prefusion conformation. In contrast, the templated two-stranded peptide immunogen is comparatively rigid and elicited a higher proportion of antibodies that recognized the native conformation of the HRC region in its prefusion state. This also explains why the antibody to the templated two-stranded HRC peptide immunogen had the weakest affinity for the postfusion conformation of HRC in the six-helix bundle construct (monomeric HRC in each of the three grooves of the six-helix bundle, Figure 14C).

Antigenicity of the Synthetic HRC Peptides

The antibody to the templated two-stranded HRC peptide immunogen binds to the prefusion coiled-coil trimer but also binds the one-stranded BSA conjugate and HRC-GCN4 construct. This observation is potentially explained by the induced fit model (*109–113*) where an initial interaction would be followed by either rearrangement of the peptide into the same conformation as that found in the coiled-coil trimer and/or conformational changes to the binding pocket in the antibody that would promote more defined complementary interactions between the peptide and antibody. The antibody to the templated two-stranded HRC peptide immunogen does not interact strongly with the HRC peptide in the highly stable postfusion conformation of the six-helix bundle where monomeric HRC peptides are individually bound in the grooves of the trimeric HRN coiled-coil. Thus, the HRC peptide in the HRC six-helix bundle construct is stabilized (with N- and C-termini in an extended conformation) and cannot adopt the more helical prefusion conformation recognized by the antibody to the templated two-stranded HRC. The antibody to the one-stranded HRC immunogen, on the other hand, can recognize the stabilized HRC trimer in the GCN4-HRC-GCN4 construct (prefusion mimic), the more flexible HRC trimer in the HRC-GCN4 construct (proposed intermediate mimic) and highly stabilized monomeric helix of HRC in the six-helix bundle construct (postfusion mimic) albeit, with different affinities. This suggests that the flexibility of the one-stranded peptide epitope induced antibodies that predominantly recognize the monomeric conformation of the HRC peptide as presented in the postfusion conformation of the six-helix bundle. The antibody to the peptide scaffold-three-stranded HRC immunogen exhibited stronger binding to the HRC-GCN4 construct (flexible HRC trimer), compared to its interactions with the GCN4-HRC-GCN4 (stabilized HRC trimer, prefusion mimic) and six-helix bundle (stabilized monomeric HRC, postfusion mimic) constructs (Figure 14). Thus, the peptide scaffold-three-stranded HRC antibody recognizes the less structured epitope presented by the HRC-GCN4 construct, which may represent an intermediate state(s) of the S protein during the fusion process.

While the induced fit model agrees well with our current observations regarding the antibodies generated from immunization with the synthetic HRC peptide immunogens, it does not explain results obtained with antibodies generated against an HRC-nanoparticle conjugate (*75*). These antibodies successfully bound the HRC region of native S protein but failed to interact with free HRC peptide. These results suggest that presentation of the HRC peptide on the nanoparticle resulted in antibodies that recognized a discontinuous epitope made up of more than one strand of the HRC trimer and therefore, could not bind or recognize the monomeric HRC peptide.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Recognition of HRC by Conformation-Specific HRC Antibodies

An NMR solution structure of the HRC region of the S protein in the absence of HRN but in the presence of the helix-inducing environment of TFE demonstrated an intrinsic tendency toward formation of a trimeric coiled-coil (*96*). The prefusion mimic of HRC, namely GCN4-HRC-GCN4, also exhibited a high helical content that was stable to thermal denaturation (Figure 13). It is important to note that the helical character of the HRC region in the GCN4-HRC-GCN4 construct is likely similarly stable in the prefusion state of the native S protein. It follows that the three highly stabilized HRC helices in the prefusion trimer would be rigid and therefore not easily induced into a conformation recognized by antibodies elicited by more flexible immunogens. This may explain why the antibody to the one-stranded peptide immunogen did not bind to the native trimeric S protein and why antibodies to the one-stranded or peptide scaffold-three-stranded immunogens bound with weaker affinity to the GCN4-HRC-GCN4 construct compared to the antibody to the templated two-stranded HRC immunogen (Figure 14). As a result, neither antibody afforded neutralization against virus entry (Figure 9) nor prevented viral transmission by cell-cell fusion (Figure 10). This line of reasoning is further supported by the observation that all three antibodies recognized the relatively flexible one-stranded and peptide scaffold-three-stranded peptide-BSA conjugates (Figure 7A and B) as well as the ability of all three antibodies to bind to the HRC-GCN4 construct (Figure 14), which is more flexible than the GCN4-HRC-GCN4 construct. Furthermore, while the most biologically effective immunogen was presented to the immune system as a two-stranded coiled-coil, the antibodies elicited by this immunogen recognize not only the two-stranded state but can also recognize the three-stranded coiled-coil found in the native S protein in the prefusion state. These results suggest that the HRC epitope is in the same conformation in both the two-stranded and three-stranded coiled-coils and that the HRC epitope recognized by the antibody to the templated two-stranded HRC peptide immunogen involves only a single strand of the HRC coiled-coil.

Principles and Applications of the Two-Stranded Coiled-Coil Template

There is an obvious need for technologies to stabilize the structure of peptide immunogens for the generation of antibodies that recognize specific conformations in native proteins. We report a peptide-based template for the generation of conformation-specific antibodies that recognize specific α-helices in native proteins. As shown in Figure 2 and 3, the template is designed as a parallel, two-stranded, α-helical coiled-coil structure that provides maximum stability through an isoleucine/leucine hydrophobic core and an interchain disulfide bridge. Surface-exposed helical residues from the relevant epitope sequence from the protein of interest are inserted into the template. The two-stranded template is used for immunization to generate polyclonal antibodies, which are specific not only for the amino acid sequence of interest but also for its α-helical conformation (*84*).

Antibodies elicited by our templated α-helical immunogens bind specifically to the surface-exposed residues of the native protein. For example, antibodies elicited by a templated HRC immunogen and a templated HRN immunogen, which have the same template hydrophobic core residues at the same heptad repeat registration but with their own surface-exposed residues from S protein, did not cross-react with the HRN and HRC antigens, respectively (Figure 15).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

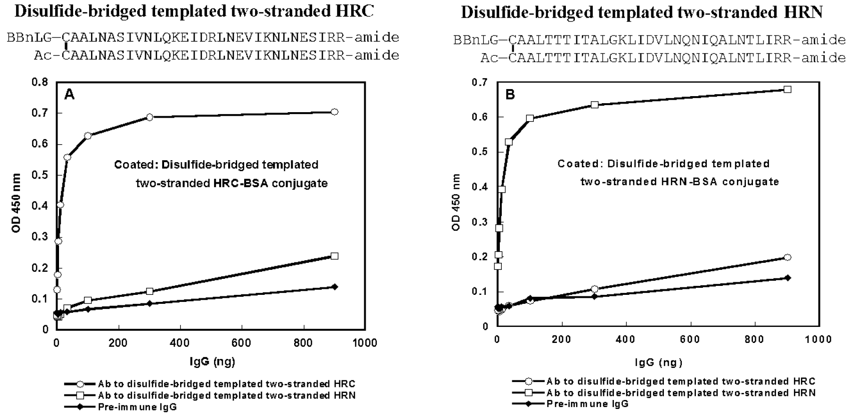


Figure 15. ELISA cross-reactivity of anti-HRC antibody against HRN and anti-HRN antibody against HRC to demonstrate specificity. Panel A: Wells were coated with disulfide-bridged two-stranded HRC-BSA conjugate. Panel B: Wells were coated with disulfide-bridged two-stranded HRN-BSA conjugate. Serial (three-fold) dilutions of the antibodies were applied to the peptide (0.2 µg/well) and the amount of bound antibodies measured by an ELISA assay. The background was estimated by the amount of antibody bound to BSA and subtracted.

There are many advantages of using this *de novo* two-stranded coiled-coil template to display helical epitopes, such as: a well-defined structural framework, the peptide immunogen can be designed to have high intrinsic stability and high solubility; it can be highly tolerant to different sequences without losing the overall three-dimensional structure; due to their small size, coiled-coil sequences can be readily prepared by chemical synthesis; it contains a sufficiently large molecular surface for antibody interaction; and the bivalent nature maximizes antigen presentation and focuses the immune response. This is also a valid technology to characterize the conformation and stability of potential helical epitopes of native proteins. These synthetic peptide immunogens will help to further our understanding, at the molecular level, of the structural requirements of immunogens to protect against viral infection.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Conclusion and Perspectives

The exact rules that dictate antigen-antibody interactions have yet to be defined. It is not surprising then that the mechanism of antibody cross-reactivity with an epitope presented by a synthetic peptide versus the full-length protein is still not understood. It seems obvious, however, that a constrained peptide that effectively mimics structural elements of the same sequence in the native protein is more likely to generate antibodies that recognize both the peptide and the native protein compared to the corresponding unstructured linear peptide.

Our templated peptide immunogens are α-helical coiled-coils that present surface exposed residues corresponding to helical regions of the proteins from which they are derived. We demonstrated here that our technology for stabilizing a synthetic peptide immunogen as an α-helical coiled-coil was an effective strategy for generating antibodies that bound the SARS-CoV S protein and neutralized the viral pathogen.

Since class I viral fusion proteins, like the S protein of SARS-CoV, are helical in nature, our technology for generating antibodies against such regions will be useful for the design of vaccines that target a wide range of viral pathogens. Furthermore, unlike traditional vaccines that typically target the immunodominant but highly mutagenic receptor binding head regions of class I viral fusion proteins, our peptide immunogens can be designed to elicit antibodies to more conserved and structurally/functionally relevant helical domains.

Currently, we are using this strategy to develop a " Universal Influenza Synthetic Peptide Vaccine " that targets the highly conserved α-helical segments of the stem region of viral hemagglutinin (HA) glycoprotein. Such a vaccine will provide broad and long lasting protection against many subtypes of influenza with different HA proteins (e.g. H1N1, H2N2, H5N1) and will not need to be changed annually to protect against virus variants that arise by antigenic drift or antigenic shift variants. Since the annual toll of seasonal influenza on humans worldwide is more than a billion cases, a " Universal Vaccine " would dramatically reduce the economic burden that results from health care costs, lost workdays and loss of life.

Overall, these results clearly demonstrate that our conformation-stabilized two-stranded coiled-coil template acts as an excellent platform to elicit α-helix-specific antibodies against highly conserved viral antigens. In the opinion of the authors, conformationally-constrained synthetic peptide vaccines will play a significant role in human health and will ultimately replace existing vaccine approaches as science identifies the protein targets and the key regions within the targets of structural and functional importance.

Materials and Methods

Peptide Synthesis

Peptides were prepared by solid-phase synthesis as previously described (*29*) using 4-benzylhydrylamine hydrochloride resin with conventional N^{α}-*t*-butyloxycarbonyl (Boc) chemistry. Following synthesis the peptides were deprotected and N-terminally acylated with acetic anhydride (Ac), benzoylbenzoic acid (BB) anhydride or Boc-*p*-amino-benzoylbenzoic acid (Abz) anhydride. The N-terminally acetylated and Abz peptides were cleaved from the resin with hydrogen fluoride (10 mL/g of resin) containing 10% anisole (v/v) and 2% 1,2-ethanediol at −4 °C for 1 h. For peptides that contained BB, thioanisole was used in place of anisole and 1,2-ethandiol was omitted. Following cleavage and removal of hydrogen fluoride, the crude peptides were washed several times with ethyl ether, extracted with 50% acetonitrile (v/v) and lyophilized. Crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a C_8 semipreparative HPLC column (Zorbax SB-C8 300 Å, 6.5 μ, 9.4 mm I.D. × 250 mm column) (*29*). A shallow gradient approach (0.1% acetonitrile/min) (*114*) was employed using an AB gradient where eluent A was 0.2% aqueous TFA and eluent B was 0.2% TFA in acetonitrile. Each peptide was characterized by amino acid analysis and electrospray mass spectrometry (*29*).

Formation of Disulfide-Bridged Templated Two-Stranded Peptide

The disulfide-bridged templated two-stranded peptide was prepared as previously described (*29*). A 0.1 mg/μL solution of 2,2′-dithiodipyridine (DTDP) was prepared using *N*,*N*-dimethylformamide. A 10 μl (3.4 μmol) aliquot of this solution was then added to a solution containing 2 mg (0.8 μmol) of the N-terminal acetylated HRC peptide in 3:1 (v/v) acetic acid/H_2O and the reaction mixture was stirred for 6 h. Distilled water (1 mL) was added and the solution was extracted with ethyl ether (3 x 500 μL). The aqueous layer was loaded onto a Sephadex G-25 desalting column conditioned with 50 mM NH_4Ac, pH 5.5 (running buffer). Fractions with a 220 nm absorbance were pooled to give the pure peptide with the thiopyridine derivative (TP) of cysteine. The HRC peptide that contained the BB moiety, the norleucine glycine linker and a free cysteine was dissolved in 8 M urea, and 50 mM NH_4Ac, pH 5.5, buffer to give a 2mg/mL solution of peptide. This peptide was added in 100 μl aliquots to the TP derivative over 30 min. The reaction was then stirred for 1 h to form the disulfide-bridged templated two-stranded peptide, which was purified by RP-HPLC as described above (*29*).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Formation of Peptide Scaffold-Three-Stranded HRC Peptide

The HRC peptide with a N-terminal cysteine (CGG-HRC peptide, Figure 5) and the peptide scaffold (BB-nLGKGKGKGRR-amide) were synthesized and purified as previously described (*29*). The three ε-amino groups from the lysine side-chains of the scaffold were reacted with the N-hydroxysuccinimide ester of bromoacetic acid (10 eq) in PBS to give three bromoacetyl moieties attached to the scaffold. Then, the peptide scaffold-three-stranded peptide was prepared by adding 3 molar equivalents of CGG-HRC peptide (25 mM in PBS) to 1 equivalent of bromoacetylated scaffold. The reaction mixture was stirred for 1 h and the reaction was monitored for completion by LC-MS. The solution was then loaded onto an analytical RP-HPLC column (4.6 mm I.D. × 250 mm Zorbax SB-C8 300 Å, 5 μ) and purified using a linear AB gradient with a gradient rate of 2% acetonitrile/min, where A is aqueous 0.2% TFA and B is 0.2% TFA in acetonitrile. Fractions deemed >95% pure by RP-HPLC were pooled and lyophilized to give the pure peptide-scaffold-three-stranded HRC peptide.

Preparation of Peptide-Carrier Protein Conjugates

Peptides used for immunization were conjugated to keyhole limpet haemocyanin (KLH) and peptides used in enzyme-linked immnosorbent assays (ELISA) were conjugated to bovine serum albumin (BSA) (*29*). The one-stranded HRC peptide-KLH conjugate was prepared by addition of the HRC peptide with a C-terminal cysteine into a solution of maleimide-activated KLH (0.5 mM in 8 M urea, PBS). Peptide-KLH conjugates of the templated two-stranded HRC and peptide scaffold-three-stranded HRC were prepared by first dissolving KLH in 8 M urea, 50 mM sodium bicarbonate, pH 8.9, to give a KLH concentration of 25 mg/mL. To 100 μL of the KLH solution was added approximately 2 mg of the templated two-stranded HRC or peptide scaffold-three-stranded HRC (BB containing) peptide such that the molar ratio was ~8:1 peptide:carrier. The peptide carrier solutions were put in quartz tubes, placed in a Rayonet photoreaction chamber (Southern New England Ultraviolet Company, Bradford, CT) and irradiated with UV light (350 nm) for 2 h. 4-Benzoylbenzoic acid (added during synthesis) served as a photo-activated linker to crosslink the peptide to the carrier protein. After reaction completion, the conjugation mixture (100 μL) was diluted to 2 mL with 2 M urea and 50 mM sodium bicarbonate, pH 8.5, then dialyzed against 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4 in PBS overnight at 4 °C. Peptide-BSA conjugates were prepared using the same procedure as for peptide-KLH conjugates. An average peptide:KLH ratio of 4:1 was determined by amino acid analysis (*29*).

Immunization Protocol

All of the animal work was carried out as previously reported (*29*) at the University of Colorado Health Sciences Center laboratory in accordance with established protocols on file. Briefly, for each immunogen, three New Zealand white rabbits were immunized at two intramuscular sites. Primary immunization

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

contained 50 μg of the keyhole limpet haemocyanin-peptide conjugate (in PBS, pH 7.4) mixed 1:1 with Freund's complete adjuvant. Secondary, tertiary and booster immunizations (at days 7, 28, and 50) also contained 50 μg of conjugate but were mixed with Freund's incomplete adjuvant. After exsanguination on day 58, serum was collected and stored at −20 °C.

Purification of IgG

Polyclonal antibodies were precipitated from sera using ammonium sulfate. Sera were diluted 1:1 into PBS and then crystalline ammonium sulfate was added to 45% saturation (0.277 g/mL) while stirring in an ice bath to precipitate the immunoglobulins. Centrifugation at 7000g was used to collect the precipitated antibodies. The pellet was resuspended in 2.5 mL of PBS and dialyzed against PBS, pH 7.4 three times. The antibody solution was then further purified on a protein G affinity column (1.5 cm I.D. × 10 cm, protein G–Sepharose 4 Fast Flow, Amersham Biosciences, Piscataway, NJ). The bound antibody was eluted from the column using 0.5 M ammonium acetate, pH 3 buffer after which the solution was immediately adjusted to pH 7–8 with ammonium hydroxide and dialyzed against PBS overnight. Subsequently, the antibody solution was concentrated to > 10 mg/mL in an Amicon concentration unit using YM30 ultrafiltration discs (Millipore Corp., Bedford, MA). The concentration of each antibody solution was determined by OD_{280} using a concentration standard IgG sample as a reference. Finally, the antibody solution was stored at −20 °C until use.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD). The CD wave scans were measured from 190 to 255 nm in benign buffer (0.1 M potassium chloride, 0.05 M potassium phosphate, pH 7.2). Temperature denaturation midpoints ($T_{1/2}$) for the peptides were determined by following the change in molar ellipticity at 222 nm from 4 to 95 °C in a 1 mm path length cell and a temperature increase rate of 1 °C/min. Ellipticity readings were normalized where 1 represents the molar ellipticity values for the fully folded species and zero equals the fully unfolded species.

ELISA Protocol

High-binding, 96-well polystyrene microplates were coated with diluted immunogens (0.01 mg/mL) using 100 mM carbonate, pH 9.6 overnight at 4 °C or 1 h at 37 °C. After removing the coating solution and washing three times with PBS, each well was blocked with 100 μL 5% BSA in PBS (37 °C, 1 h). The sera or Protein G purified antibodies (IgG) were diluted in 1% BSA in PBS and added to each well and incubated at 37 °C for 1 h. The sample solution was removed and washed three times by PBS containing 0.1% Tween20. Next, horseradish peroxidase (HRP) conjugated anti-mouse IgG (goat) (diluted 1:10,000) was added to each well and incubated for 1 h at 37 °C. The sample solution was then removed and the wells were washed three times with PBS containing 0.1%

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Tween20. A solution of 2,2’-Azino-di-3-ethyl-benthiazoline-sulfonic acid (0.6 mg/mL) in 10 mM citrate, pH 4.2 containing 0.1% H_2O_2 was added to each well and then the plates were read at 450 nm.

Binding of HRC Antibodies to S Protein on Cell Surface

The HEK 293T cells were grown to 70-80% confluence in T75 flasks and transfected with 25 µg of pcDNA3.1-SARS SΔ19 using polyethylenimine (PEI). The pcDNA3.1-SARS SΔ19 plasmid has a deletion of the last 19 amino acids of S protein, which removes the ER/Golgi retention signal. After incubation (40 h), cells were detached with PBS containing 1 mM EDTA. Cells were washed twice with PBS containing 2% normal goat serum (NGS) and placed into 96-well plates at $1x10^5$ cells per well. Cells were then incubated with 100 µL of antibodies (1:250 dilution in PBS containing 2% NGS) on ice for 1 h. After washing twice with PBS containing 2% NGS, cells were incubated with 100 µL of goat anti-rabbit IgG conjugated with phycoerythrin (PE) on ice for 1 h. Cells were washed twice again with PBS containing 2% NGS and antibody binding was analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, USA).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Inhibition of Entry of SARS-S Pseudotyped Retrovirus by HRC Antibodies

To produce SARS-S pseudotyped retrovirus, equal molar ratio of three plasmids, murine stem cell virus (MSCV), pcDNA3.1 gag-pol, and pcDNA3.1-SARS SΔ19, were co-transfected into HEK293T cells using PEI. After 40 h, viruses were harvested by centrifugation (1,000g, 5 min) and cell debris was removed by filtration through 0.45 µm filters. Viruses were diluted 10-fold into medium containing 0.5 mg/mL of antibodies (total rabbit IgG). After 30 min incubation at 37 °C, the virus-antibody mixture was added onto HEK293T cells stably expressing recombinant human angiotensin-converting enzyme 2 (293/hACE2), the receptor for SARS-CoV. After 24 h incubation, cells were detached with trypsin and fixed with 1% paraformaldehyde in PBS, then analyzed by flow cytometry according to green fluorescent protein (GFP) expression in infected cells.

Inhibition of SARS-S Mediated Syncytia Formation by HRC Antibodies

HEK293T cells were transfected with pcDNA3.1-SARS SΔ19. After 40 h incubation, cells were detached with trypsin. After being washed twice with medium to remove trypsin, cells were overlaid with 293/hACE2 cells at a ratio of 1:3 in presence of 1mg/mL of anti-HRC antibodies. After 3 h, cells were fixed with crystal violet fixation solution and S-mediated cell fusion (syncytia formation) was monitored by light microscopy.

Expression and Purification of HRC-GCN4 and GCN4-HRC-GCN4

The SARS-CoV HRC domain consisting of residues 1150-1185 was subcloned and fused with modified GCN4 sequence (at C-terminus or N-and

C-terminus) into the BamHI/HindIII restriction sites of a modified pQE30 expression vector (Qiagen). The resulting constructs, termed His-PG-HRC-GCN4 and His-PG-GCN4-HRC-GCN4), consisted of a N-terminal polyhistidine tag followed by protein G (the IgG-binding domain of streptococcus protein G), a tobacco etch virus (TEV) cleavage site (sequence, ENLYFQGS) for removal of the expression tag. Protein expression was achieved by growing *Escherichia coli* strain BL-21 in LB medium and induced with 0.8 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37 °C. The two fusion proteins were purified from the soluble fraction using a Ni^{2+} fast flow Sepharose column (Qiagen). The protein was then cleaved using TEV protease. RP-HPLC was used to purify HRC-GCN4 and GCN4-HRC-GCN4 from the cleavage solutions. The constructs were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a C_{18} semipreparative HPLC column (Zorbax SB-C18 300 Å, 5 μ, 9.4 mm I.D. × 250 mm column) using an AB gradient of 30-70%B (0.2% acetonitrile/min) where eluent A was 0.2% aqueous TFA and eluent B was 0.2% TFA in acetonitrile. The purity and identity of HRC-GCN4 and GCN4-HRC-GCN4 were confirmed by SDS-PAGE and LC-MS.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

References

1. Miller, M.; Barrett, S.; Henderson, D. A. In *Disease Control Priorities in Developing Countries*, 2nd ed.; Jamison, D. T., Breman, J. G., Measham, A. R., Alleyne, G., Claeson, M., Evans, D. B., Jha, P., Mills, A., Musgrove, P., Eds.; World Bank: Washington, DC, 2006; p 1163−1176.
2. Beyer, W. E.; Palache, A. M.; Kerstens, R.; Masurel, N. *Eur. J. Clin. Microbiol. Infect Dis.* **1996**, *15*, 65–70.
3. Davis, A. R.; Nayak, D. P.; Ueda, M.; Hiti, A. L.; Dowbenko, D.; Kleid, D. G. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 5376–5380.
4. Arnon, R. *Mol. Immunol.* **1991**, *28*, 209–215.
5. Cooper, J. A.; Hayman, W.; Reed, C.; Kagawa, H.; Good, M. F.; Saul, A. *Mol. Immunol.* **1997**, *34*, 433–440.
6. Lin, Y. S.; Lin, C. F.; Fang, Y. T.; Kuo, Y. M.; Liao, P. C.; Yeh, T. M.; Hwa, K. Y.; Shieh, C. C.; Yen, J. H.; Wang, H. J.; Su, I. J.; Lei, H. Y. *Clin. Exp. Immunol.* **2005**, *141*, 500–508.
7. Czub, M.; Weingartl, H.; Czub, S.; He, R.; Cao, J. *Vaccine* **2005**, *23*, 2273–2279.
8. Olsen, C. W. *Vet. Microbiol.* **1993**, *36*, 1–37.
9. Chen, Z.; Zhang, L.; Qin, C.; Ba, L.; Yi, C. E.; Zhang, F.; Wei, Q.; He, T.; Yu, W.; Yu, J.; Gao, H.; Tu, X.; Gettie, A.; Farzan, M.; Yuen, K. Y.; Ho, D. D. *J. Virol.* **2005**, *79*, 2678–2688.
10. Kam, Y. W.; Kien, F.; Roberts, A.; Cheung, Y. C.; Lamirande, E. W.; Vogel, L.; Chu, S. L.; Tse, J.; Guarner, J.; Zaki, S. R.; Subbarao, K.; Peiris, M.; Nal, B.; Altmeyer, R. *Vaccine* **2007**, *25*, 729–740.
11. He, Y.; Li, J.; Heck, S.; Lustigman, S.; Jiang, S. *J. Virol.* **2006**, *80*, 5757–5767.
12. Carrat, F.; Flahault, A. *Vaccine* **2007**, *25*, 6852–6862.

13. Barbey-Martin, C.; Gigant, B.; Bizebard, T.; Calder, L. J.; Wharton, S. A.; Skehel, J. J.; Knossow, M. *Virology* **2002**, *294*, 70–74.
14. Wiley, D. C.; Wilson, I. A.; Skehel, J. J. *Nature* **1981**, *289*, 373–378.
15. Bommakanti, G.; Citron, M. P.; Hepler, R. W.; Callahan, C.; Heidecker, G. J.; Najar, T. A.; Lu, X.; Joyce, J. G.; Shiver, J. W.; Casimiro, D. R.; ter Meulen, J.; Liang, X.; Varadarajan, R. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 13701–13706.
16. Gocnik, M.; Fislova, T.; Mucha, V.; Sladkova, T.; Russ, G.; Kostolansky, F.; Vareckova, E. *J. Gen. Virol.* **2008**, *89*, 958–967.
17. Ekiert, D. C.; Bhabha, G.; Elsliger, M. A.; Friesen, R. H.; Jongeneelen, M.; Throsby, M.; Goudsmit, J.; Wilson, I. A. *Science* **2009**, *324*, 246–251.
18. Sui, J.; Hwang, W. C.; Perez, S.; Wei, G.; Aird, D.; Chen, L. M.; Santelli, E.; Stec, B.; Cadwell, G.; Ali, M.; Wan, H.; Murakami, A.; Yammanuru, A.; Han, T.; Cox, N. J.; Bankston, L. A.; Donis, R. O.; Liddington, R. C.; Marasco, W. A. *Nat. Struct. Mol. Biol.* **2009**, *16*, 265–273.
19. Ekiert, D. C.; Friesen, R. H.; Bhabha, G.; Kwaks, T.; Jongeneelen, M.; Yu, W.; Ophorst, C.; Cox, F.; Korse, H. J.; Brandenburg, B.; Vogels, R.; Brakenhoff, J. P.; Kompier, R.; Koldijk, M. H.; Cornelissen, L. A.; Poon, L. L.; Peiris, M.; Koudstaal, W.; Wilson, I. A.; Goudsmit, J. *Science* **2011**, *333*, 843–850.
20. Purcell, A. W.; Zeng, W.; Mifsud, N. A.; Ely, L. K.; Macdonald, W. A.; Jackson, D. C. *J. Pept. Sci.* **2003**, *9*, 255–281.
21. Dudek, N. L.; Perlmutter, P.; Aguilar, M. I.; Croft, N. P.; Purcell, A. W. *Curr. Pharm. Des.* **2010**, *16*, 3149–3157.
22. Lerner, R. A. *Adv. Immunol.* **1984**, *36*, 1–44.
23. WHO 2010.
24. Purcell, A. W.; McCluskey, J.; Rossjohn, J. *Nat. Rev. Drug Discovery* **2007**, *6*, 404–414.
25. Zompra, A. A.; Galanis, A. S.; Werbitzky, O.; Albericio, F. *Future Med. Chem.* **2009**, *1*, 361–377.
26. Tosh, P. K.; Boyce, T. G.; Poland, G. A. *Mayo Clin. Proc.* **2008**, *83*, 77–84.
27. Siegrist, C. A. *J. Comp. Pathol.* **2007**, *137* (Suppl 1), S46–50.
28. Kao, D. J.; Hodges, R. S. *Chem. Biol. Drug Des.* **2009**, *74*, 33–42.
29. Tripet, B.; Kao, D. J.; Jeffers, S. A.; Holmes, K. V.; Hodges, R. S. *J. Struct. Biol.* **2006**, *155*, 176–194.
30. Dunshea, F. R.; Colantoni, C.; Howard, K.; McCauley, I.; Jackson, P.; Long, K. A.; Lopaticki, S.; Nugent, E. A.; Simons, J. A.; Walker, J.; Hennessy, D. P. *J. Anim. Sci.* **2001**, *79*, 2524–2535.
31. https://animalhealth.pfizer.com/sites/pahweb/US/EN/Products/Pages/Improvest.aspx.
32. Dyson, H. J.; Sayre, J. R.; Merutka, G.; Shin, H. C.; Lerner, R. A.; Wright, P. E. *J. Mol. Biol.* **1992**, *226*, 819–835.
33. Dyson, H. J.; Lerner, R. A.; Wright, P. E. *Annu. Rev. Biophys. Biophys. Chem.* **1988**, *17*, 305–324.
34. Siddiqui, M. Z.; Sharma, A. K.; Kumar, S.; Kumar, A.; Bhat, P. N. *Int. J. Biol. Macromol.* **1994**, *16*, 259–263.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

35. Gariepy, J.; Mietzner, T. A.; Schoolnik, G. K. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8888–8892.
36. Jemmerson, R. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9180–9184.
37. Palmenberg, A. *Nature* **1987**, *329*, 668–669.
38. Satterthwait, A. C.; Arrhenius, T.; Hagopian, R. A.; Zavala, F.; Nussenzweig, V.; Lerner, R. A. *Philos. Trans. R Soc. London, Ser. B* **1989**, *323*, 565–572.
39. McInnes, C.; Sonnichsen, F. D.; Kay, C. M.; Hodges, R. S.; Sykes, B. D. *Biochemistry* **1993**, *32*, 13432–13440.
40. Erlanger, B. F. *Pharmacol. Rev.* **1973**, *25*, 271–280.
41. Sundaram, R.; Dakappagari, N. K.; Kaumaya, P. T. *Biopolymers* **2002**, *66*, 200–216.
42. Edwards, R. J.; Singleton, A. M.; Boobis, A. R.; Davies, D. S. *J. Immunol. Methods* **1989**, *117*, 215–220.
43. Briand, J. P.; Muller, S.; Van Regenmortel, M. H. *J. Immunol. Methods* **1985**, *78*, 59–69.
44. Wang, T. T.; Tan, G. S.; Hai, R.; Pica, N.; Ngai, L.; Ekiert, D. C.; Wilson, I. A.; Garcia-Sastre, A.; Moran, T. M.; Palese, P. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 18979–18984.
45. Aref, N. E.; Saeed, A. M. *J. Immunol. Methods* **2011**, *366*, 100–105.
46. Hoogerhout, P.; Kamphuis, W.; Brugghe, H. F.; Sluijs, J. A.; Timmermans, H. A.; Westdijk, J.; Zomer, G.; Boog, C. J.; Hol, E. M.; van den Dobbelsteen, G. P. *PLoS One* **2011**, *6*, e19110.
47. Kantele, A.; Hakkinen, M. P.; Zivny, J.; Elson, C. O.; Mestecky, J.; Kantele, J. M. *Clin. Dev. Immunol.* **2011**, *2011*, 614383.
48. Partidos, C. D.; Stanley, C. M.; Steward, M. W. *J. Gen. Virol.* **1991**, *72* (Pt 6), 1293–1299.
49. Lairmore, M. D.; DiGeorge, A. M.; Conrad, S. F.; Trevino, A. V.; Lal, R. B.; Kaumaya, P. T. *J. Virol.* **1995**, *69*, 6077–6089.
50. Frangione-Beebe, M.; Albrecht, B.; Dakappagari, N.; Rose, R. T.; Brooks, C. L.; Schwendeman, S. P.; Lairmore, M. D.; Kaumaya, P. T. *Vaccine* **2000**, *19*, 1068–1081.
51. Sundaram, R.; Lynch, M. P.; Rawale, S. V.; Sun, Y.; Kazanji, M.; Kaumaya, P. T. *J. Biol. Chem.* **2004**, *279*, 24141–24151.
52. Garrett, J. T.; Rawale, S.; Allen, S. D.; Phillips, G.; Forni, G.; Morris, J. C.; Kaumaya, P. T. *J. Immunol.* **2007**, *178*, 7120–7131.
53. Kaumaya, P. T.; Foy, K. C.; Garrett, J.; Rawale, S. V.; Vicari, D.; Thurmond, J. M.; Lamb, T.; Mani, A.; Kane, Y.; Balint, C. R.; Chalupa, D.; Otterson, G. A.; Shapiro, C. L.; Fowler, J. M.; Grever, M. R.; Bekaii-Saab, T. S., III; Carson, W. E., III *J. Clin. Oncol.* **2009**, *27*, 5270–5277.
54. Harris, J. R.; Markl, J. *Micron* **1999**, *30*, 597–623.
55. Houston, M. E., Jr.; Gannon, C. L.; Kay, C. M.; Hodges, R. S. *J. Pept. Sci.* **1995**, *1*, 274–282.
56. Precheur, B.; Bossus, M.; Gras-Masse, H.; Quiniou, E.; Tartar, A.; Craescu, C. T. *Eur. J. Biochem.* **1994**, *220*, 415–425.
57. Leonetti, M.; Pillet, L.; Maillere, B.; Lamthanh, H.; Frachon, P.; Couderc, J.; Menez, A. *J. Immunol.* **1990**, *145*, 4214–4221.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

58. Cuniasse, P.; Thomas, A.; Smith, J. C.; Thanh, H. L.; Leonetti, M.; Menez, A. *Biochemistry* **1995**, *34*, 12782–12789.
59. Kaumaya, P. T.; VanBuskirk, A. M.; Goldberg, E.; Pierce, S. K. *J. Biol. Chem.* **1992**, *267*, 6338–6346.
60. Moseri, A.; Tantry, S.; Sagi, Y.; Arshava, B.; Naider, F.; Anglister, J. *Virology* **2010**, *401*, 293–304.
61. Hadley, E. B.; Testa, O. D.; Woolfson, D. N.; Gellman, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 530–535.
62. Burkhard, P.; Stetefeld, J.; Strelkov, S. V. *Trends Cell Biol.* **2001**, *11*, 82–88.
63. Bianchi, E.; Finotto, M.; Ingallinella, P.; Hrin, R.; Carella, A. V.; Hou, X. S.; Schleif, W. A.; Miller, M. D.; Geleziunas, R.; Pessi, A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 12903–12908.
64. Russell, R. J.; Kerry, P. S.; Stevens, D. J.; Steinhauer, D. A.; Martin, S. R.; Gamblin, S. J.; Skehel, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 17736–17741.
65. McFarlane, A. A.; Orriss, G. L.; Stetefeld, J. *Eur. J. Pharmacol.* **2009**, *625*, 101–107.
66. Hodges, R. S.; Saund, A. K.; Chong, P. C.; St-Pierre, S. A.; Reid, R. E. *J. Biol. Chem.* **1981**, *256*, 1214–1224.
67. O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber, T. *Science* **1991**, *254*, 539–544.
68. Hodges, R. S. *Curr. Biol.* **1992**, *2*, 122–124.
69. Zhou, N. E.; Zhu, B. Y.; Kay, C. M.; Hodges, R. S. *Biopolymers* **1992**, *32*, 419–426.
70. Hodges, R. S. *Biochem. Cell Biol.* **1996**, *74*, 133–154.
71. Louis, J. M.; Nesheiwat, I.; Chang, L.; Clore, G. M.; Bewley, C. A. *J. Biol. Chem.* **2003**, *278*, 20278–20285.
72. Joyce, J. G.; Hurni, W. M.; Bogusky, M. J.; Garsky, V. M.; Liang, X.; Citron, M. P.; Danzeisen, R. C.; Miller, M. D.; Shiver, J. W.; Keller, P. M. *J. Biol. Chem.* **2002**, *277*, 45811–45820.
73. De Filette, M.; Martens, W.; Roose, K.; Deroo, T.; Vervalle, F.; Bentahir, M.; Vandekerckhove, J.; Fiers, W.; Saelens, X. *J. Biol. Chem.* **2008**, *283*, 11382–11387.
74. Raman, S.; Machaidze, G.; Lustig, A.; Aebi, U.; Burkhard, P. *Nanomedicine* **2006**, *2*, 95–102.
75. Pimentel, T. A.; Yan, Z.; Jeffers, S. A.; Holmes, K. V.; Hodges, R. S.; Burkhard, P. *Chem. Biol. Drug Des.* **2009**, *73*, 53–61.
76. Fernandez-Carneado, J.; Grell, D.; Durieux, P.; Hauert, J.; Kovacsovics, T.; Tuchscherer, G. *Biopolymers* **2000**, *55*, 451–458.
77. Correia, B. E.; Ban, Y. E.; Holmes, M. A.; Xu, H.; Ellingson, K.; Kraft, Z.; Carrico, C.; Boni, E.; Sather, D. N.; Zenobia, C.; Burke, K. Y.; Bradley-Hewitt, T.; Bruhn-Johannsen, J. F.; Kalyuzhniy, O.; Baker, D.; Strong, R. K.; Stamatatos, L.; Schief, W. R. *Structure* **2010**, *18*, 1116–1126.
78. Ofek, G.; Guenaga, F. J.; Schief, W. R.; Skinner, J.; Baker, D.; Wyatt, R.; Kwong, P. D. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 17880–17887.
79. McLellan, J. S.; Correia, B. E.; Chen, M.; Yang, Y.; Graham, B. S.; Schief, W. R.; Kwong, P. D. *J. Mol. Biol.* **2011**, *409*, 853–866.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

80. Walsh, S. T.; Cheng, H.; Bryson, J. W.; Roder, H.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5486–5491.
81. Barthe, P.; Rochette, S.; Vita, C.; Roumestand, C. *Protein Sci.* **2000**, *9*, 942–955.
82. Kobs-Conrad, S.; Lee, H.; DiGeorge, A. M.; Kaumaya, P. T. *J. Biol. Chem.* **1993**, *268*, 25285–25295.
83. Miceli, R.; Myszka, D.; Mao, J.; Sathe, G.; Chaiken, I. *Drug Des. Discovery* **1996**, *13*, 95–105.
84. Lu, S. M.; Hodges, R. S. *J. Biol. Chem.* **2002**, *277*, 23515–23524.
85. Helmke, S. M.; Lu, S. M.; Harmon, M.; Glasford, J. W.; Larsen, T. D.; Kwok, S. C.; Hodges, R. S.; Perryman, M. B. *J. Mol. Recognit.* **2006**, *19*, 215–226.
86. Xu, Y.; Lou, Z.; Liu, Y.; Pang, H.; Tien, P.; Gao, G. F.; Rao, Z. *J. Biol. Chem.* **2004**, *279*, 49414–49419.
87. Supekar, V. M.; Bruckmann, C.; Ingallinella, P.; Bianchi, E.; Pessi, A.; Carfi, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17958–17963.
88. Tripet, B.; Howard, M. W.; Jobling, M.; Holmes, R. K.; Holmes, K. V.; Hodges, R. S. *J. Biol. Chem.* **2004**, *279*, 20836–20849.
89. Eckert, D. M.; Kim, P. S. *Annu. Rev. Biochem.* **2001**, *70*, 777–810.
90. White, J. M.; Delos, S. E.; Brecher, M.; Schornberg, K. *Crit. Rev. Biochem. Mol. Biol.* **2008**, *43*, 189–219.
91. Matsuyama, S.; Ujike, M.; Morikawa, S.; Tashiro, M.; Taguchi, F. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 12543–12547.
92. Simmons, G.; Reeves, J. D.; Rennekamp, A. J.; Amberg, S. M.; Piefer, A. J.; Bates, P. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4240–4245.
93. Yang, Z. Y.; Huang, Y.; Ganesh, L.; Leung, K.; Kong, W. P.; Schwartz, O.; Subbarao, K.; Nabel, G. J. *J. Virol.* **2004**, *78*, 5642–5650.
94. Simmons, G.; Gosalia, D. N.; Rennekamp, A. J.; Reeves, J. D.; Diamond, S. L.; Bates, P. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 11876–11881.
95. Inoue, Y.; Tanaka, N.; Tanaka, Y.; Inoue, S.; Morita, K.; Zhuang, M.; Hattori, T.; Sugamura, K. *J. Virol.* **2007**, *81*, 8722–8729.
96. McReynolds, S.; Jiang, S.; Rong, L.; Caffrey, M. *J. Magn. Reson.* **2009**, *201*, 218–221.
97. Buchholz, U. J.; Bukreyev, A.; Yang, L.; Lamirande, E. W.; Murphy, B. R.; Subbarao, K.; Collins, P. L. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9804–9809.
98. McReynolds, S.; Jiang, S.; Guo, Y.; Celigoy, J.; Schar, C.; Rong, L.; Caffrey, M. *Biochemistry* **2008**, *47*, 6802–6808.
99. Hakansson-McReynolds, S.; Jiang, S.; Rong, L.; Caffrey, M. *J. Biol. Chem.* **2006**, *281*, 11965–11971.
100. Yan, Z.; Tripet, B.; Hodges, R. S. *J. Struct. Biol.* **2006**, *155*, 162–175.
101. Zhou, N. E.; Monera, O. D.; Kay, C. M.; Hodges, R. S. *Protein Pept. Lett.* **1994**, *15*, 238–243.
102. Ngai, S. M.; Sonnichsen, F. D.; Hodges, R. S. *J. Biol. Chem.* **1994**, *269*, 2165–2172.
103. Parker, J. M. R. a. H. R. S. *J. Protein Chem.* **1985**, *3*, 465–478.
104. Parker, J. M. R. a. H. R. S. *J. Protein Chem.* **1985**, *3*, 479–489.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

105. Gans, P. J.; Lyu, P. C.; Manning, M. C.; Woody, R. W.; Kallenbach, N. R. *Biopolymers* **1991**, *31*, 1605–1614.
106. Sonnichsen, F. D.; Van Eyk, J. E.; Hodges, R. S.; Sykes, B. D. *Biochemistry* **1992**, *31*, 8790–8798.
107. Luzzago, A.; Felici, F.; Tramontano, A.; Pessi, A.; Cortese, R. *Gene* **1993**, *128*, 51–57.
108. Hoess, R. H.; Mack, A. J.; Walton, H.; Reilly, T. M. *J. Immunol.* **1994**, *153*, 724–729.
109. Getzoff, E. D.; Geysen, H. M.; Rodda, S. J.; Alexander, H.; Tainer, J. A.; Lerner, R. A. *Science* **1987**, *235*, 1191–1196.
110. Rini, J. M.; Schulze-Gahmen, U.; Wilson, I. A. *Science* **1992**, *255*, 959–965.
111. Colman, P. M.; Laver, W. G.; Varghese, J. N.; Baker, A. T.; Tulloch, P. A.; Air, G. M.; Webster, R. G. *Nature* **1987**, *326*, 358–363.
112. Bhat, T. N.; Bentley, G. A.; Fischmann, T. O.; Boulot, G.; Poljak, R. J. *Nature* **1990**, *347*, 483–485.
113. Wedemayer, G. J.; Patten, P. A.; Wang, L. H.; Schultz, P. G.; Stevens, R. C. *Science* **1997**, *276*, 1665–1669.
114. Chen, Y.; Mant, C. T.; Hodges, R. S. *J. Chromatogr., A* **2007**, *1140*, 112–120.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch006

Chapter 7

Defensins in Viral Infection

Rachna Shah and Theresa L. Chang[*]

Public Health Research Institute and Department of Microbiology
and Molecular Genetics, University of Medicine and Dentistry
of New Jersey-New Jersey Medical School, 225 Warren Street,
Newark, NJ 07103, U.S.A.
[*]E-mail: changth@umdnj.edu

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Defensins are antimicrobial peptides important for innate immune responses. Defensins can positively or negatively modulate infection by both enveloped and non-enveloped viruses. The effect of defensins on viral infection varies significantly depending on the specific virus, defensin and cell type in vitro. This chapter focuses on the interplay between human defensins and viral infection. Understanding the functions of defensins in viral infection may provide insights into developing novel anti-viral preventative or therapeutic strategies.

Introduction

The innate immune system offers the first line of defense before the development of an adaptive immune response (*1*, *2*). Defensins are antimicrobial peptides important for the innate immune responses. These peptides protect the host from the invasion of pathogens through their antimicrobial activity and by acting as immunomodulators (*3*). While in vitro and in vivo functions of defensins against bacteria have been long recognized, the role of defensins in modulating viral infection began to flourish in recent years. It was thought that defensins primarily target enveloped virus by disrupting the lipid membrane of the envelope similar to their anti-bacterial activities. However, recent knowledge indicates that defensin function is complex and may positively or negatively modulate infection by both enveloped and non-enveloped viruses. Several excellent review articles have provided detailed information with respect to mammalian defensins and their immunological roles (*4*–*9*). This chapter will primarily focus on the

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

interplay between mammalian defensins and viral infection, the underlying molecular mechanisms, and the roles of human defensins in viral pathogenesis and transmission.

Overview of Human Defensins

Classification

Human defensins are cationic peptides, consisting of approximate 30 amino acids in length, with β-sheet structures stabilized by three disulfide bonds between the cysteine residues. Based on their disulfide bond linkages, human defensins are classified into two subfamilies: α-, and β- defensins (reviewed in (*4*, *8*, *10*). The linkages of Cys residues in α-defensins are Cys^1–Cys^6, Cys^2–Cys^4, Cys^3–Cys^5, whereas in β-defensins the linkages are Cys^1–Cys^5, Cys^2–Cys^4, Cys^3–Cys^6 (Figure 1). Despite variation in sequences and disulfide bond linkages, both families have similar structures (*11–14*). In humans, there are 6 α−defensins: human neutrophil peptides 1-4 (HNPs 1-4) and human α-defensins 5 and 6 (HD5 and HD6). Six human β-defensins (HBD1, -2, -3, -4, -5, -6) have been identified and characterized (*8*, *15*, *16*), although gene-based analysis indentifies an additional 28 human β-defensins (*17*).

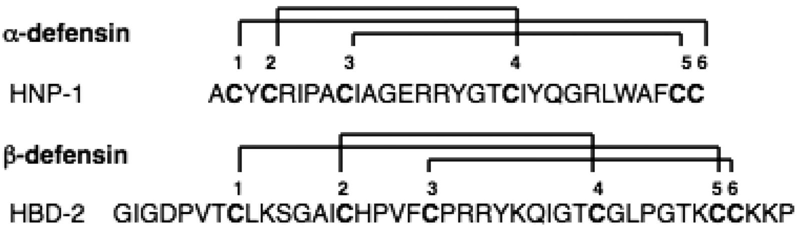

α-defensin
1 2 3 4 5 6
HNP-1
ACYCRIPACIAGERRYGTCIYQGRLWAFCC
β-defensin
1 2 3 4 5 6
HBD-2
GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP

Figure 1. Disulfide pairing of cysteine residues of α- and β-defensins.

The α−defensins are synthesized as a pre-pro-peptide, which comprises of an amino (N)-terminal signal sequence, an anionic propiece, and a carboxyl (C) terminal mature peptide (*4*). HNPs 1-4 are synthesized in promyelocytes, neutrophil precursor cells in the bone marrow, and the mature peptide is stored in primary granules of neutrophils (*4*). Unlike HNPs, HD5 is released as a propeptide that is processed extracellularly (*18*, *19*). An additional class of mammalian defensins is the θ-defensins with a circular structure originally found in rhesus monkeys (*20*). Six θ-defensins have been found in leukocytes and neutrophils of rhesus macaques: rhesus θ-defensin-1-6 (RTDs 1-6) (*20–23*). Human RNA transcripts homologous to the rhesus θ-defensin gene (DEFT for defensin theta) are found in human bone marrow but contain a premature stop codon in the signal sequence (*24*). Retrocyclin, an artificially synthesized circular peptide based on the sequence of the mature peptide that would be encoded by the human θ-defensin pseudogene, displays antiviral activity *in vitro* (*25*).

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Structure−Function Relationship

The structure of defensins is crucial for the chemotactic and antiviral activities, but it may or may not affect the antibacterial activities depending on specific defensins or bacteria. For examples, disulfide bonds have been shown to be dispensable for antibacterial functions of HNP1, HBD3, and cryptdin-4, a mouse Paneth cell α−defensin (*26–28*). However, properly folded HBD3 is important for its chemotactic activity (*27*). Similarly, HNPs 1-3 or θ-defensins, after treatment with the reducing agents, lose the ability to directly affect the virion (*29*, *30*). Interestingly, a recent study indicates that, after reduction of disulfide bonds, HBD1 becomes a potent antimicrobial peptide against opportunistic pathogenic fungus *Candida albicans* and against commensal Gram-positive bacteria such as *Bifidobacterium* and *Lactobacilli* (*31*). In vitro, the thioredoxin (TRX) system can reduce HBD-1. Additionally, TRX co-localizes with reduced HBD-1 in human epithelia (*31*). Authors suggest that reduced HBD-1 shield the healthy epithelium against colonization of commensal bacteria and pathogenic fungi (*31*), although the clinical function of reduced HBD-1 remains to be determined.

HD5 and HD6 linear analogs lost their HIV enhancing effect (*32*). Disulfide bonds of cryptdin-4 are required for protection from proteolysis by matrix metalloproteinase-7 (*26*). Analysis of electropositive charge of cryptdin-4 and rhesus myeloid α-defensin 4 (RMAD-4) revealed that substitution of Arg with Lys attenuated antibacterial activity in cryptdin-4 but not in RMAD-4, and primary structure of α-defensins determined the function Arg to Lys analogs (*33*). Importantly, recent studies indicate that linear or unstructured defensins retain their antibacterial activity in a bacterial strain-dependent manner (*34*). The hydrophobicity and/or amphiphilicity rather than cationicity plays an important role in the antibacterial activities of α-defensins (*33*, *35*). For example, Trp-26 in HNP1 has been shown to be crucial for the ability of defensins to kill *S. aureus*, inhibit anthrax lethal factor and bind HIV gp120 (*35*). Overall, the knowledge with respect to the impact of the defensin structure on the effect of defensins on viral infection is limited. Further investigations using defensin analogs with mutation of specific residues are required to shed light on the relationship between the structure and function of defensins on viral infection.

Cell Sources and Tissue Distribution

Human defensins are produced mainly by leukocytes and epithelial cells. HNPs 1-3, differing in one amino acid at the N-terminus (*36*), were first isolated from polymorphonucleated neutrophilic leukocytes, and account for 30-50% of total protein in azurophil granules of neutrophils (*37*). No gene encoding HNP2 was found and thus it is thought to be a proteolytic product of HNP1 or HNP3. HNP4 comprising less than 2% of defensins in neutrophils has a relatively distinct sequence but similar structure with HNPs1-3 (*4*, *38*). While neutrophils produce the highest amount of HNPs, these peptides can be found in other immune cells including natural killer cells, B cells, γδ T cells, and monocytes/macrophages, immature dendritic cells (*39*, *40*). Cells can absorb and internalize HNPs intracellularly (*41–43*), underling the complexity in defining true HNP producing cells. HNPs have been detected in placenta, spleen, thymus, intestinal mucosa, saliva, and cervical mucus plugs (*40*, *44–46*). Elevation of HNPs has been reported in genital mucosa in individuals with *N. gonorrhoeae* (GC)*, T. vaginalis, or C. trachomatis* (CT) infection, bacterial vaginosis and endometritis (*47*) (*48–50*).

Although leukocyte α−defensins are conserved evolutionally and have been isolated from many species including human, rabbits, rats, guinea pigs and hamsters, mice do not express neutrophil α-defensin (*4*). Mice express many cryptdins, enteric α-defensins, in intestinal Paneth cells (*4*, *7*). Similarly, HD5 and HD6 are produced predominantly by intestinal Paneth cells in humans (*51*), although HD5-transgenic mice are markedly resistant to oral challenge with virulent *Salmonella typhimurium*, indicating that human and mouse enteric defensins have distinct functions (*52*). In rhesus macaque, six Paneth cell defensins have been identified and their coding sequences are distinct from HD5 and HD6 (*53*). HD5 can be found in other tissues such as the salivary glands, the female genital tract and the inflamed large bowel (*45*, *54–56*), whereas HD6 is over-expressed in colon cancer (*57*, *58*).

HBDs 1-3 are primarily expressed by epithelial cells but also found in hematopoietic cells including peripheral blood mononuclear cells (PBMCs), monocytes, macrophages, plasmocytoid dendritic cells (pDCs) and monocyte-derived dendritic cells (MDDCs) (*4*, *8*, *59–62*). Expression of HBDs 4-6 is limited to specific tissues (*15*). While HBD1 is often constitutively expressed, production of HBD1 mRNA and peptides is found in pDCs and mononcytes in response to viral exposure (*60*). Expression of HBD2 and HBD3 can be induced by viruses, bacteria, microbial products and pro-inflammatory cytokines, such as tumor-necrosis factor (TNF) and interleukin-1 (IL-1) (*4*, *63–66*). HBD1, HBD2 and HBD3 have been detected in various epithelial tissues (*45*, *67*, *68*). Additionally, both human α- and β-defensins have been found in breast milk (*69*, *70*).

Immunological and Biological Functions of Defensins

Defensins have a wide range of functions in modulating innate and adaptive immunity (*8*) as well as metabolisms and angiogenesis (*9*, *71–75*). Both HNPs and HBDs exhibit chemotactic activity for T cells, monocytes and immature DCs and can induce production of cytokines and chemokines (*8*) (*76*, *77*). HNP1 also regulates the release of IL-1β and enhances phagocytosis (*78*, *79*). In addition, HNPs upregulate the expression of CC-chemokines and IL-8 in macrophages and epithelial cells, respectively (*80*, *81*). Intestinal α-defensin HD5 can induce IL-8 in vitro (*82*). HBDs1-3 recruit memory T cells and immature DCs through binding to CCR6, the receptor for the CC-chemokine ligand 20 (CCL20; also known as MIP3α) (*83*, *84*). HBD2 can up-regulate IL-6, IL-8, IL-10, MCP-1, IL-1β, MIP-1β and RANTES in PBMCs (*85*), and exhibits multiple activities on mast cells, including induction of cell migration, degranulation and prostaglandin D_2 production (*86*). Murine β-defensin-2 can recruit bone-marrow-derived immature DCs through CCR6 and can induce DC maturation through TLR4

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

(*87*). HBD3 activates antigen presenting cells (DCs and monocytes) via TLR1/2 (*88*). HBD3 induces the expression of IL-1α, IL-6, IL-8, CCL18 and TNF-α in monocyte-derived macrophages, and recruits monocytes/ macrophages through CCR2 (*89*). HBD3 also binds to CXCR4, one of the main HIV co-receptors, and competes with its natural ligand – stromal-derived factor (SDF-1) (*90*). In contrast to SDF-1, HBD3 does not induce calcium flux and ERK1/ERK2 phosphorylation. HBD2 and HBD3 have been shown to down-regulate CXCR4 in PMBCs and cell lines expressing CXCR4 in the absence of serum (*91*, *92*). In addition, HBD2 and HBD3, and their mouse orthologs, mBD4 and mBD14 can induce CCR6-independent chemotaxis by interacting with CCR2 (*93*).

Defensins can bind to other host proteins to modulate immune or metabolic functions (*9*). HNPs bind to low-density lipoprotein receptor-related proteins and interact with protein kinase Cα and β, leading to decreased smooth muscle contraction in response to phenylephrine (*94*). HNPs also interact with adrenocorticotrophic hormone receptors and heparan sulfate proteoglycan (HSPG) to modulate other biological activities (*95*, *96*). HNP1 inhibits the activity of conventional PKC isoforms in a cell-free system (*97*). This PKC inhibitory activity appears to be important for HNP1-mediated inhibition of HIV replication in primary $CD4^+$ T cells (*98*) and suppression of influenza A virus (IAV) in lung epithelial cells (*99*). Additionally, HNP1 blocks the classical and lectin pathways of complement activation by binding to complement C1q and mannose-binding lectin, respectively (*100*, *101*). As defensins display multiple biological functions, the role of defensins in viral-associated metabolic diseases or cancers in addition to viral transmission and pathogenesis warrants further investigation.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Regulation of Defensins

HNP1 and HNP3 can be transcriptionally regulated by the binding of CCAAT/ enhancer-binding protein (C/EBP α) to C/EBP/c-Myb sites in the HNP promoter (*102*, *103*). In response to bacterial infection, high concentrations of HNPs (mg/ ml) are present in neutrophil phagosomes as a result of the fusion of granules and phagocytic vacuoles of neutrophils (*4*, *104*). Stimulation with chemokines, FCγ receptor cross-linking, or phorbol myristate acetate can cause the release of HNPs (*41*, *105–107*). Outer membrane protein A of *Klebsiella pneumoniae* and flagellin of *Escherichia coli*, which activate toll-like receptors (TLRs) 2 and 5, respectively, trigger the release of HNPs 1-3 by the $CD3^+CD56^+$ natural killer T cells (*106*). Direct interaction of *Mycobacterium bovis* BCG with eosinophils induces the production and release of HNPs 1-3 in a TLR2 dependent manner (*108*).

HD5, highly abundant in the small intestine, is constitutively expressed by Paneth cells but can be found in the colon of patients with inflammatory bowel disease (*109*) (*52*, *110*). In patients with ileal Crohn's disease (CD), a chronic mucosal inflammation, a NOD2 (nucleotide-binding oligomerization domain containing 2) mutation is associated with a pronounced reduction in HD5 production (*111*). There is an association between reduced expression of the Wnt signaling transcription factor Tcf-4 protein and a decrease in HD5 and

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

HD6 expression in small intestine from patients with ileal CD, although this association is independent of the NOD2 genotype (*112*). HD5 is induced at the genital mucosa in patients with bacterial vaginosis, GC and CT infections (*19*, *47*). HD5 and HD6 can be induced in response to GC infection in cervicovaginal epithelial cells (*32*).

HBDs 1-3 can be induced by cytokines, TLR activation or viral exposure but the underlying mechanisms appear to be distinct from each other (*16*, *66*). HBD2 can be induced by TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2 signaling in various epithelial cells and keratinocytes (*113–117*). TLR3 activation induces HBD1 and HBD2 expression in uterine epithelial cells and keratinocytes (*60*, *118*) and increases HBD2 and HBD3 expression in bronchial epithelial cells (*64*). In oral epithelium, TLR2 and NOD1/2 ligands synergistically activate nuclear factor-κB (NF-κB) and induce HBD2 gene expression (*119*). Stimulation of TLR2 and TLR4 with peptidoglycans and lipopolysaccharides can induce HBD2 expression in keratinocytes and vaginal epithelial cells (*116*, *117*).

Cytokines also play important roles in the regulation of HBD expression. Induction of HBD2 by IL-17A is mediated by the PI3K pathway and MAPK pathway to activate NF-κB in airway epithelial cells, whereas regulation of HBD2 by NF-κB is not dependent on the PI3K pathway in bronchial epithelial cell, (*120–122*), indicating that specific pathways involved in regulation of HBDs are cell type dependent. In human keratinocytes, TNF-α induces gene expression of HBD2 but not HBD3 in a dose-dependent manner (*123*). Unlike HBD-2, HBD-3 mRNA is preferentially stimulated by IFN-γ but not by TNF-α indicating specific HBD regulation in response to specific cytokines.

Effect of Viral Infection/Exposure on Defensin Expression

Both enveloped DNA and RNA viruses such as herpes simplex virus-1 (HSV-1), influenza virus, and Sendai virus increase expression of HBD1 in pDC, monocytes and epithelial cells (*60*). UV-irradiated HSV-1 but not UV-irradiated influenza virus can induce HBD gene expression in monocytes (*60*). HIV-1 induces mRNA expression of HBD2 and HBD3 but not HBD1 in normal human oral epithelium and cells, even in the absence of HIV-1 replication (*92*). However, a recent study reported that both X4- and R5-tropic viruses cannot induce HBD2 gene expression in the MatTeck oral tissue model nor primary gingival epithelial cells (*124*). Additionally, high concentrations of X4 virus HIV-1_{Lai} block TNF-α induced HBD2 gene expression by 50% (*124*). Expression of HBD2 and HBD3 but not HBD1 is induced in bronchial epithelial cells exposed to human rhinovirus (*63*, *64*). In mice with infection by IAV, gene expression of murine β-defensin 3 and 4 (orthologs of HBD2) is induced in upper and lower airways, and active replication of rhinovirus is required for HBD gene induction (*125*). Induction of HBD2 in response to human rhinovirus infection is mediated by NF-κB activation but is independent of IL-1 (*63*). Furthermore, a similar profile of HBD gene expression is induced by TLR3 activation, suggesting that intracellular double-stranded RNA generated during replication of rhinovirus may be involved in the upregulation of HBD2 and HBD3 expression (*63*, *64*). Induction of HBD2 plays a role in innate antiviral response against human respiratory syncytial virus (RSV) in lung epithelial cells that is TNFα-mediated NF-κB dependent but type I interferon independent (*126*). Productive RSV infection activates NF-κB and induces TNFα, resulting in induction of HBD2 that is required for TNF-α-mediated anti-RSV activity. Similar to infection of IAV in mice, RSV infection induces expression of mBD3 and mBD4 in the lung.

Effect of Defensins on Viral Infection

HNP1 was originally reported to have a direct effect on several enveloped viruses but not on non-enveloped viruses (*29*). It has a potent direct inhibitory effect on HSV-1 and HSV-2, a moderate direct effect on vesicular stomatitis virus and influenza virus, and little effect on cytomegalovirus (*29*). However, it is known now that defensins exhibit anti-viral activities against both enveloped and non-enveloped viruses (see below). The exact mechanism of direct inactivation of the virion by defensins is not entirely clear. Recent evidence indicates that defensins modulate viral infection through multiple mechanisms. The effect of defensins on viral infection in vitro is dependent on defensins, viruses, and target cells. Defensins can modulate viral infection through a direct interaction with virus or through interactions with potential target cells. Table 1 and Table 2 summarize the activities of defensins on infection by both enveloped and non-enveloped viruses, respectively. It is important to note that one cannot extrapolate the in vitro activity of defensins to their in vivo effect. Increasing evidence indicates that specific defensins can control viral infection and disease progression by modulating immune responses despite of the lack of anti-viral activities in vitro.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

HIV

In contrary to the traditional role of defensins to defend host against pathogens, recent studies indicate that specific defensins can inhibit or enhance HIV infection. With respect to anti-HIV activities of defensins, these peptides have a dual role in antiviral activity by acting on the virus or the target cells.

The in vitro functions of defensins appear to be affected by factors such as serum and salt that may determine defensin functions depending on the sites (e.g. mucosal surfaces versus blood). In addition, results can vary depending on the source of defensin (e.g. recombinant vs synthetic peptides with or without proper folding). Serum and salt conditions did alter the direct effect of HNPs and HBDs on the virion (*29*, *92*, *98*) but are not required for the chemotactic effects of defensins (*77*, *83*). As some defensins (e.g. HNPs but not HD5 or HD6) at high concentrations are known to cause cytotoxicity in the absence of serum, which is associated with changes in cell membrane permeability but can be abolished by the presence of serum (*127*), defensin-mediated cytotoxicity may partially account for the antiviral effect (*42*).

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Table 1. Effect of defensins on infection by enveloped viruses

Viruses	***Defensins***	***Effect***	***Mechanism***	***References***
Enveloped viruses				
HIV	HNP1	Inhibit	Inactivates virion	(*42*), (*98*)
	HNP1, HNP2	Inhibit	Upregulates CC-chemokines production by macrophages	(*81*)
	HNPs1-3	Inhibit	Bind to gp120 and CD4, block fusion	(*129*), (*134*)
	HNP1	Inhibit	Blocks viral nuclear import and transcription	(*98*)
	HNP4	Inhibit	Binds to gp120 and CD4 (lectin-independent)	(*132*), (*134*)
	HD5, HD6	Enhance	Enhance viral entry and attachment	(*32*), (*135*)
	Cryptidin-3	Enhance	N/A	(*53*)
	HBD1	No effect	N/A	(*92*), (*137*)
	HBD2	Inhibit	Blocks early RT product formation,	(*137*)
		Inhibit	Induction of APOBEC3G	(*138*)
	HBD2, HBD3	Inhibit	Downregulate CXCR4 expression	(*92*)
	Retrocyclin	Inhibit	Blocks viral entry	(*25*), (*139*)
	Retrocyclin	Inhibit	Binds to gp120 and CD4	(*25*), (*30*),(*134*),(*139*)
	Retrocyclin 1	Inhibit	Blocks viral fusion	(*140*)
	RTD 1-3	Inhibit	Binds to gp120 and CD4	(*134*)
	Rat NP-1	Inhibit	Blocks post entry	(*128*)
	Rabbit NP-1	Inhibit	Inhibits post entry	

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Viruses	*Defensins*	*Effect*	*Mechanism*	*References*
Enveloped viruses				
HSV1	HNP1	Inhibit	Inactivates virion	(*29*)
	Rabbit NP-1	Inhibit	Blocks viral fusion, entry and post-entry steps	(*142*)
HSV2	HNP1	Inhibit	Inactivates virion (weak activity)	(*29*)
	HNPs 1-3	Inhibit	Inhibit viral entry	(*143*)
	HNPs 1-3, HD5	Inhibit	Inhibit post-entry steps	(*144*)
	HNP4, HD6, HBD3	Inhibit	Inhibit viral attachment and entry	(*144*)
	Rabbit NP-1	Inhibit	Blocks viral fusion, entry and post-entry steps	(*142*)
	Retrocyclin-2	Inhibit	Inhibits viral attachment and entry	(*143*)
IAV	HNP1	Inhibit	Inactivates virion (weak activity)	(*29*), (*99*)
	HNP1, HNP2, HD5	Inhibit	Aggregate virus, enhances viral clearance by neutrophils	(*78*), (*145*)
	HNP1	Inhibit	Interferes with cell signaling	(*99*)
	HBD1 (in vivo)	Inhibit	Modulate immune responses	(*60*)
	HBD3, retrocyclin-2	Inhibit	Inhibits viral fusion	(*146*)
RSV	HBD2	Inhibit	Inhibits viral entry, disrupts viral envelope	(*126*)
PIV 3	Sheep BD1	Inhibit	N/A	(*148*)
	HBD6 (in vivo)	Enhance		(*149*)

Continued on next page.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Table 1. (Continued). Effect of defensins on infection by enveloped viruses

Viruses	***Defensins***	***Effect***	***Mechanism***	***References***
Enveloped viruses				
Vaccinia virus	HNP1	No effect	N/A	(*152*)
	HBD3	Inhibit	N/A	(*152*),(*153*)
VSV	HNP1	Inhibit	Inactivates virion	(*29*)
CMV	HNP1	Inhibit	Inactivates virion (weak activity)	(*29*)
SARS-COV	RTD-1	No effect	N/A	(*151*)
	RTD-1 (in vivo)	Inhibit	Modulates immune response	(*151*)

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Table 2. Effect of defensins on infection by non-enveloped viruses

Viruses	***Defensins***	***Effect***	***Mechanism***	***References***
Non-enveloped viruses				
Polyomavirus BKV	HNP1, HD5	Inhibit	Aggregates virus, prevents binding	(*154*)
	HBD1, HBD2	No effect		(*154*)
HAdV-A, -B, -C, -E	HNP1, HD5	Inhibit	Stabilizes virus capsid, prevents uncoating	(*155*), (*156*), (*157*), (*158*), (*160*)
	HBD1	Inhibit	N/A	(*157*)
	HBD1	No effect	N/A	(*156*)
	HBD2	No effect	N/A	(*155*),(*156*),(*158*)
HAdV-D,-F	HD5, HNP1	Enhance/ No effect	N/A	(*156*),(*160*)
	HBD1, HBD2	No effect	N/A	(*156*)
Papillomavirus	HNP1, HD5	Inhibit	Restrain virus in endosomes	(*161*)

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Synthetic guinea-pig, rabbit and rat α-defensins was first reported in 1993 to block infection by HIV-1 X4 in transformed CD4$^+$ T cells in the presence of serum after viral entry (*128*). The 50% inhibitory concentrations (IC_{50}) of these peptides range from 9-12 μM. HNPs1-3 inhibit HIV infection through multiple mechanisms (*42*, *129–131*). The IC_{50} varies from 0.5-60 μM depending on the source of HNPs and HIV infection assay systems. HNPs1-3 all have similar activities against HIV primary isolates (*132*), in contrast to their differential chemotactic activities on monocytes, where HNP3 has no effect (*133*). They can inhibit HIV-1 replication by direct interaction with the virus as well by affecting multiple steps of HIV life cycle (*42*, *98*, *129*, *131*, *134*). In the absence of serum, HNP1 can directly inactivate the virus prior to infection of a cell (*98*). HNPs1-3 can act as lectins and bind to HIV envelope glycoprotein gp120 and to CD4 with high affinity (*134*). The binding to gp120 is strongly attenuated by serum, thus accounting for the loss of the direct virion effect in the presence of serum. Interestingly, in contrast to HNPs1-3, HNP4 acts in a lectin-independent manner and does not bind to CD4 or HIV gp120 (*132*, *134*). However, HNP4 inhibits HIV replication more effectively than HNP1, -2 and -3 (*132*).

In the presence of serum and at non-cytotoxic concentrations (low dose), HNP1 blocks HIV-1 infection by acting on primary CD4+ T cells at the steps of nuclear import and transcription (*98*). The post-entry inhibitory effect of HIV infection is involved in PKC signaling pathways and occurs in primary CD4$^+$ T cells and macrophages but not in several transformed T-cell lines (*98*, *131*). In the presence of serum, HNP1 did not affect expression of cell-surface CD4 and HIV-coreceptors on primary CD4+ T cells (*98*), whereas HNP2 down-regulates CD4 expression in the absence of serum (*129*). HNPs block HIV-mediated cell-cell fusion and the early steps of HIV infection by interacting with HIV-1 gp120 and CD4 through their lectin-like properties (*129*). In macrophages, HNP1 and HNP2 upregulate the expression of CC-chemokines, which could contribute to inhibition of HIV through competition for receptors (*81*). CC-chemokines can also induce the release of HNPs from neutrophils by degranulation (*107*). While both HNPs and CC-chemokines exhibit anti-HIV activities in vitro, their ability of recruiting immature dendritic cells and CD4+ T cells, which are HIV target cells, may lead to an increase in the susceptibility to HIV at the mucosa.

The effect of other α-defensins, including human Paneth cell defensins (HD5 and HD6), mouse Paneth cell defensins (cryptdin-3 and cryptdin-4), and rhesus macaque myeloid α-defensins (RMAD3 and RMAD4) on HIV infection has been tested (*32*, *53*). At high concentrations associated with cytotoxicity, RMAD4 blocks HIV replication, whereas cryptdin-3 enhances HIV replication. While HD5 did not exhibit any effect on X4 HIV-1_{LAI} infection of transformed CD4+ T cell lines (*53*), HD5 and HD6 at 10-50 μg/ml significantly enhanced infectivity of HIV-1 in primary CD4+ T cells and HeLa-CD4-CCR5 cells (*32*). The enhancing effect of HD5 and HD6 is more pronounced with R5 virus compared with X4 virus, indicating a potential role of mucosal transmission of HIV as R5 virus is preferentially transmitted during primary infection. HD5 and HD6 enhance HIV infection by promoting HIV attachment (*135*). These defensins interfere with anti-HIV activities of polyanionic microbicides including PRO2000, cellulose sulfate and carrageenan (*136*), which have failed to protect women against HIV infection in large-scale clinical trials. HD5 and HD6 also block anti-HIV activity of entry (TAK799) and fusion (T-20) inhibitor when inhibitors are only present during HIV attachment but not infection (*135*). These results highlight the importance of understanding the role of mucosal innate effectors in the efficacy of microbicides during the pre-clinical screening. HD5 and HD6 block anti-HIV activities of soluble glycosaminoglycans including heparin, chondroitin sulfate and dextran sulfate, although heparin at a high concentration diminishes the HIV enhancing effect of HD5 but not HD6 (*135*). The degree of the HIV enhancing effect of HD5 but not HD6 is increased in heparinase-treated cells. Together with the results of soluble glycosaminoglycans, heparin or heparan sulfate appears to compete with HD5, but not HD6, for binding to HIV. HD5 and HD6 appear to enhance HIV infectivity through distinct mechanisms.

The anti-HIV activities of HBD2 (IC_{50} ~0.2-0.4 μM) and HBD3 (IC_{50} ~0.4-0.8 μM) have been demonstrated under different experimental conditions such as the presence of serum and the source of defensins (*92*, *137*). The binding of defensins to cellular membrane and HIV virion has been demonstrated by electron microscopy, although membrane disruption is not apparent (*92*). HBD2 does not affect viral fusion but inhibits the formation of early reverse transcribed HIV DNA products (*137*). Further delineation of the anti-HIV effect of HBD2 on the target cells reveals that HBD2 inhibits HIV infection by inducing the intrinsic restriction factor APOBEC3G (*138*). There are conflicting results on the downregulation of expression of HIV co-receptors by HBDs. Sun *et al.* (*137*) demonstrated that HBD1 and HBD2 do not modulate cell-surface HIV co-receptor expression by primary CD4+ T cells, whereas Quinones-Mateu *et al.* (*92*) showed that HBD2 and HBD3 down-regulated surface CXCR4 but not CCR5 expression by PBMCs at high salt conditions and in the absence of serum. Rohri et al show that HBD2:Ig and HBD3:Ig fusion proteins bind to HEK293 cells expressing CCR2 but not CXCR4 (*93*). Seidel et al demonstrate that HBD2 blocks both X4 and R5 virus and down-regulate CXCR4 but not CD4 or CCR5 in both PBMCs and GHOST cell lines expressing CCR5 and CXCR4 at 37°C but not at 4°C (*91*). Interestingly, HBD2 is constitutively expressed in healthy adult oral mucosa but the level seems to be diminished in HIV-infected individuals (*137*).

Retrocyclins (IC_{50} ~0.5-10μM), and RTD1, -2 and -3 (IC_{50} ~0.45-1.8 μM) act as lectins and can inhibit HIV entry (*25*, *30*, *134*, *139*). Retrocyclin and RTD1,-2 and -3 inhibit several HIV-1 X4 and R5 viruses including primary isolates (*30*, *134*, *139*). Unlike α- and β-defensins, retrocyclin does not appear to directly inactivate the HIV virion although it is not clear whether the experiments reported to date were preformed under serum-free condition (*25*). Retrocyclin does however bind to HIV gp120 as well as CD4 with high affinity, which is consistent with inhibition of viral entry (*139*). This high-binding affinity to glycosylated gp120 and CD4 is mediated through interactions with their *O*-linked and *N*-linked sugars (*30*). Serum strongly reduces their binding to gp120 (*134*). RTD1 bind directly to the C-terminal heptad repeat of HIV envelope protein gp41, blocking formation of the six helix bundle required for fusion (*140*). Studies on retrocyclin-1 analogues indicate that modification of this peptide can enhance its potency against HIV *in vitro* (*141*).

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

HSV

Several defensins, including HNPs1-4, HD5, HD6, and HBD3, θ defensins (RTD and retrocyclin) and a rabbit defensin (NP1), have anti-viral activity against HSV-1 and HSV-2 (*142–144*). In contrast, HBD1 and HBD2 do not exhibit anti-HSV-2 activity (*60*, *144*). HNP1 has a direct effect on HSV-1 virions, which is abolished in the presence of serum (*29*). Anti-HSV-2 of HNPs1-3 and retrocyclin 2 was first reported by inhibiting viral attachment and entry but not steps following entry (*143*). However, the follow-up study indicates that HNPs 1-3 block the post-entry events (*144*). HNP-4, HD6, and HBD3 act primarily by preventing binding and entry, whereas HD5 inhibited HSV-2 replication after viral entry (*144*). With the exception of HNP4, α-defensins and θ-defensins interact with the *O*- and *N*-linked glycans of HSV-2, indicating that defensins may be acting as lectins to prevent HSV-2 gB from interacting with its receptor HSPG (*143*).

NP1, a rabbit α-defensin, has more positively charged amino acid residues than HNPs (*6*). It has a direct effect on HSV-1 and HSV-2 virions, and inhibits HSV replication at the steps of fusion and entry as well as post-entry steps (*142*).

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Influenza Virus

HNPs1-3 inhibit IAV through multiple mechanisms. While the direct effect of HNPs on the IAV particles is moderate (*29*), HNPs block various strains of IAV by acting on the target cells through interference of cell signaling (*99*) or by aggregating virus particles followed by promoting viral clearance by neutrophils (*78*, *145*). HNP1, HNP2 and HD5 but not HBD2, and HBD3 enhance the uptake of IAV by neutrophils (*78*). HBD3 blocks hemagglutinin (HA)-mediated viral fusion (*146*). In murine model, IAV-mediated mortality is significantly increased in the murine β-defensin 1 (mBD1) knock-out mice compared to wild-type mice, suggesting the role of mBD1 in IAV pathogenesis (*60*). HNPs also modulate anti-IAV activities of other innate effectors such as surfactant protein D (SP-D) by binding to SP-D and resulting in interference with the hemagglutination-inhibiting activity of SP-D (*145*) and reduction of neutrophil H_2O_2 production in response to SP-D-treated IAV (*78*). A subsequent study shows that HBDs, HD6 and HNP4 bind weakly to SP-D, whereas HNPs and retrocyclins bind SP-D with high affinity (*147*). In contrast to HNP1 and HNP2, RCs do not block SP-D anti-IAV activity.

Retrocyclin-2 blocks the step of viral fusion mediated by the viral HA proteins (*146*). In a similar manner, it inhibits fusion mediated by other viral proteins such as baculovirus gp64 and Sindbis (alphavirus) E1 proteins. By acting as a lectin, retrocyclin-2 interferes with viral-mediated fusion by crosslinking and immobilizing cell membrane glycoproteins. Accordingly, pre-treatment of either HA-expressing cells or target cells with retrocyclin-2 inhibits fusion. Similar to retrocyclin-2, HBD3 exhibits a lectin-like property and has an inhibitory effect on HA-mediated fusion and membrane protein mobility. These results suggest a common mechanism to account for a broad range of activity of an innate immune response against viruses that utilize a common pathway of membrane fusion.

Parainfluenza Virus

Respiratory syncytial virus (RSV), as well as parainfluenza virus types 1-4 (PIV-1-4), members of the paramyxoviridae family, are major causes of respiratory diseases, particularly in young children. HBD2 but not HBD1 inhibits the entry of RSV and disrupts RSV envelope (*126*). In vivo, induction of expression of sheep β-defensin-1 and other antimicrobial proteins, such as surfactant protein A (SP-A) and SP-D, correlates with a decrease in PIV-3 viral replication in neonatal lambs (*148*). Adenovirus-mediated HBD6 expression increases neutrophil recruitment and inflammation in the lungs of neonatal lambs (*149*). Interestingly, PIV-3 infection of neonatal lambs is enhanced during the treatment with adenovirus-mediated gene therapy and expression of HBD6 further exacerbates PIV-3 infection.

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)

SARS-CoV, a single-stranded, positive-sense RNA enveloped virus, emerged in 2002 and infected more than 8000 individuals with an approximate 10% mortality rate (*150*). Elevation of HNPs is found in blood from SARS-CoV-infected patients (*150*). Examination of antiviral activity of RTD-1 against SARS-CoV indicates that RTD-1 has no direct effect on the virus (*151*). Interestingly, RTD-1 pre-treatment prevents lethal infection in mice and RTD-1 treatment reduces pulmonary pathology during SARS-CoV infection. This protective effect is achieved by suppressing immune responses. For example, RTD-1 treated mice with SARS-CoV infection have significant reduction in the levels of RANTES, MIP-1α, MCP-1, IL-1α, IL-1β, and IL-6 compared to SARS-CoV-infected mice without RTD-1 treatment. This study highlights the important role of defensins as immunomodulators in controlling viral pathogenesis.

Vaccinia Virus

Human cathelicidin LL37, another important antimicrobial peptide, blocks vaccinia viral infection through direct inactivation of virion (*152*). In contrast to LL37, HNP1 has no effect on vaccinia viral infection in vitro. HBD3 but not HBD1 and HBD2 exhibit anti-viral activity against vaccinia virus (*152*, *153*). Expression of HBD3 is induced in primary keratinocytes in response to vaccinia virus infection. Importantly, IL-4 and IL-13, frequently induced in patients with atopic dermatitis who are excluded from smallpox vaccination, down-regulated vaccinia virus-mediated HBD3 induction, suggesting that a deficiency in HBD3 may increase the susceptibility of patients with atopic dermatitis to vaccinia virus infection after smallpox vaccination (*153*).

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Non-Enveloped Viruses

Defensins block infection of non-enveloped viruses via multiple mechanisms. HNP and HD5 but not HBD1 and HBD2 inhibit infection of BK virus, a polyomavirus, by targeting an early event in the viral lifecycle (*154*). HD5 inhibits BKV by acting on the virion as HD5 treatment of BKV reduces viral attachment to cells, whereas treatment of vero cells with HD5 does not alter viral infection. HD5 binds to BKV and colocalizes BKV in cells. Transmission electron microscopy analysis reveals HD5-induced aggregation of virions. HD5 also inhibited infection of cells by other related polyomaviruses including SV40 and JCV virus.

HNP1 and HD5 inhibit human adenoviral (HAdV) infection of lung and conjunctival epithelial cells (*155–158*). HD5 inhibits an early step in HAdV entry (*158*). HNP1 and HD5 block HAdV infection by stabilizing the virus capsid, thereby preventing uncoating and virus-mediated endosome penetration (*158, 159*). The anti-HAdV activity of HNP1 and HD5 is virus species specific and the activity is correlated with defensin binding to the capsid. The cryoEM analysis of the HAdV and HD5 complex indicates that HD5 interacts with the exposed surfaces of three major capsid proteins: hexon, penton base and fiber, suggesting that the multiple interacting sites lead to enhanced virion stability (*160*).

HNPs do not have a direct effect on the virions of several non-enveloped viruses, including echovirus and reovirus (*29*), whereas HBD2 does not directly inactivate rhinovirus (*63*). However, defensins may act on infected cells and suppress non-enveloped viral replication after viral entry. Using pseudoviruses carrying a green fluorescent protein, HNP1 and HD5 inhibit various papillomavirus types (*161*). Defensins are active against HPB16 pseudotyped virus in pre-treated HeLa cells or when defensin are added few hours after viral entry. These defensins do not affect initial binding of the viron and endocytosis but block virion escape from endosomes.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Clinical Aspects of Defensins

The levels of defensins are frequently altered in patients with infections or other diseases as defensins have been suggested to be components of 'alarmins' released by innate immune cells such as neuntrophils and dendritic cells to modulate immune responses (*162*). For example, elevation of HNP1 gene expression has been associated with dengue shock syndrome in children and young adults (*163*). Induction of HBD2 and HBD3 is found in HPV-associated-anal skin lessons in both HIV-positive and HIV-negative men who have sex with men (*164*), although the direct effect of HPV on induction of HBDs remains to be determined. The defensin levels in HIV-infected patients with different stages of diseases have been investigated (see below), although several studies have small sample sizes in addition to the lack of specific virologic and immunologic information of patients.

Role of Defensins in HIV Pathogenesis and Transmission

The Levels of Human Defensins in Healthy Donors

Depending on the sample collection methods and analytical approaches, the levels of defensins can be varied from one report to another. In addition, defensins have been found to interact with other cellular proteins in plasma (*96*, *165*, *166*), which may affect the measurement of defensin levels by ELISA. In healthy donors, the plasma concentration of HNPs1-3 is ranging from ~150-500 ng/ml (*167*). The level of HNPs in cervicovaginal fluid from healthy women ranges from 250 ng/ml to 5 ug/ml varying among different reports (*48*, *168*). The levels of defensins in the plasma or at the mucosal are frequently elevated in patients with infections or diseases (*71*, *169*). For examples, defensin levels in plasma from patients with sepsis reach 900-170,000 ng/ml (*170*). Concentrations of HD5 protein ranging from 1 to 50 µg/ml have been reported in diluted vaginal fluid from healthy women (*47*, *56*). The levels of HNPs in the saliva from healthy donors range from 1 to 10 µg/ml, whereas the level of HBDs 1-2 range from undetectable to 33 ng/ml (*171*). The presence of $CaCl_2$ at 250 mM in ELISAs can overcome masking by endogenous components of body fluid, the mean values of HBD2 in healthy donors are 9.5 ng/ml in saliva and 3.42 µg/g of total proteins in vaginal specimens, whereas the mean values of HBD3 are 326 ng/ml in saliva and 103 µg/g of total proteins in vaginal specimens (*172*).

Human α-Defensins

The importance of defensins in HIV pathogenesis was first suggested by the report indicating that HNPs 1-3 account for the soluble anti-HIV activity of $CD8^+$ T cells isolated from patients infected with HIV but remaining free of AIDS for a prolonged period (long-term nonprogressors, LTNPs) (*130*). HNPs1-3 were detected in the media of stimulated $CD8^+$ T cells from normal healthy controls and LTNPs but not from HIV progressors. Subsequent studies on the cell source of defensins revealed that HNPs were probably produced by co-cultured monocytes and residual granulocytes of allogenic normal donor irradiated PBMCs that were used as feeder cells, but they were not produced by the $CD8^+$ T cells themselves (*42*, *43*). Using similar co-culture systems, levels of HNPs1-3 were measured in $CD8^+$ T-cell supernatants and cervical-vaginal mononuclear cells derived from HIV-exposed seronegative individuals, HIV-infected patients, and normal controls (*173*). Higher levels of HNPs were found in CD8+ T cells from HIV-exposed seronegative individuals and HIV patients compared to normal controls. D'Agostino et al. recently demonstrated higher levels of HNPs in plasma from HIV-infected patients than healthy donors (*174*). Using a co-culture system with radiated PBMCs, higher levels of HNPs in CD8+ T cells were found in patients with HIV infection compared to the healthy donors, and the intracellular HNP levels were further increased in stimulated CD8+ T cells. The intracellular level of HNPs in neutrophils is higher in HIV-infected patients than healthy donors. There is no significant difference in the plasma level of HNPs

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

in HIV-infected patients with or without antiviral treatment (ART). However, reduction of HNPs in CD8+ T cell was found in HIV-infected patients on ART. Interestingly, this reduction in the HNP level was not found in HIV-infected patients on ART with virologic failure. In contrast to the report by D'Agostino et al., Rodriguez-Garcia et al. did not observe any association between plasma levels of HNPs and immunologic or virolgoic parameters (*175*). This report also described an increase in HNPs1-3 in dendritic cells, differentiated in vitro, in HIV controller but not non-controllers compared to healthy controls. While it was suggested that increased HNPs1-3 production by dendritic cells in HIV-infected patients is associated with slower disease progression, analysis of specific immune cell subsets without further manipulation is needed to clarify the role of HNPs in HIV disease progression.

There is a correlation between the abundance of several anti-HIV proteins, including HNPs1-3 and cell-associated HIV replication in lymphoid follicles compared with extrafollicular lymphoid tissue (*176*). Expression of these antiviral proteins is significantly lower in the follicular region, where HIV replication is concentrated, compared with the extrafollicular regions in lymph nodes from HIV-positive individuals.

The association between production of HNPs1-3 in breast milk and transmission of HIV has also been investigated (*177*). In a case-controlled study of HIV-positive women, levels of HNPs in breast milk are positively correlated with HIV RNA copy number in breast milk, which was a strong predictor of transmission. However, after adjusting for breast milk HIV copy number, higher levels of HNPs in breast milk were associated with a decreased incidence of intrapartum or postnatal HIV transmission. Bosire and colleagues performed similar studies to determine correlates of HNPs in breast milk and transmission risk in HIV-1-infected pregnant women in Nairobi followed for 12 months postpartum with their infants (*178*). Analysis of breast milk from these women at month 1 postpartum demonstrated that women with detectable HNPs and significantly higher mean breast milk HIV-1 RNA levels than women with undetectable HNPs. Increased concentrations of HNPs in breast milk are also associated with subclinical mastitis and increased CC-chemokines in breast milk. Interestingly, in contrast to the report by Kuhn et al (*177*), the level of defensins are not associated with vertical transmission, indicating a complex interplay between innate effectors, inflammation and HIV transmission.

Cationic peptides including defensins are required for anti-HIV activity of vaginal fluid from healthy women (*179*). While it is well established that sexual transmitted infections (STIs) significantly increase the likelihood of HIV transmission (*180–184*) and that levels of defensins including HNPs, HBDs and HD5 in genital fluid, are elevated in patients with STIs (*19*, *48–50*), the role of defensins in HIV transmission remains to be clarified. Studies using a cohort of HIV uninfected sex workers in Kenyan demonstrated the association between an increase in HNPs and LL-37 levels in the IgA-depleted cervicovaginal secretions from women with bacterial STIs and increased in HIV acquisition, despite that cervicovaginal secretions with high levels of HNPs and LL-37 exhibited anti-HIV activity in vitro (*168*). However, the concentrations of HNPs and LL-37 are below the reported IC_{50} in vitro, raising the question regarding the contribution

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

of defensins in anti-HIV activity of cervicovaginal secretions from these patients. This study underscores the complex role of specific defensins in HIV transmission at the vaginal mucosa and the urgent need to define the effect of elevated innate effectors on immune responses contributing to enhanced HIV acquisition.

The role of paneth cell defensins in SIV pathogenesis has been recently investigated in macaque models (*185*). Expression of rhesus enteric α-defensins (REDs) was increased in response to SIV infection. Additionally, decreased RED protein levels correlate with enteric opportunistic infection and advanced SIV disease. Because the primary sequences of RED and HD5 differ, it is not clear whether REDs in macaques could represent HD5 in humans.

HBDs

Polymorphisms in the *DEFB1* gene (coding for HBD1) have been associated with disease susceptibility (*186–188*) (*189*, *190*). Significant correlations between the single-nucleotide polymorphism (SNPs) -44C/G and -20G/A in 5' untranslated region of *DEFB1* and a risk of perinatal transmission of HIV-1 in Italian and Brazilian populations have been reported (*191*, *192*). The SNP -52G/G genotype is associated with reduced HIV-1 RNA in breast milk, but not in plasma In Mozabican HIV-infected women (*193*). Interestingly, the functional analysis of promoter indicates that these SNPs suppress expression (*194*). Studies on the role of HBD1 in mother-to-child transmission of HIV indicated that the -52G/G genotype and the -44/-52G haplotype exhibited a protective role against HIV infection in children, whereas the -52G/G genotype and the -44G/-52G haplotype were associated with low levels of HIV plasma viremia and a lower risk of maternal HIV transmission in mothers (*195*). Although HBD1 does not exhibit any effect on HIV infection in vitro, the presence of SNP may modulate the immune response by down-regulation of HBD1.

The role of defensins in protection against HIV infection has been studied in HIV-exposed seronegative (ESN) individuals. ESN expressed significantly greater mRNA copy numbers of HBD2 and 3 in oral mucosa than healthy controls, while no difference in mRNA copy numbers of HBD-1, 2 and 3 in vaginal/endocervical mucosa was observed between ESN and controls (*196*). In addition, homozygosity for the A692G polymorphism is significantly more frequent in ESN than in seropositive individuals (*196*).

Sequence analysis of θ-defensin pseudogenes in ESN female sex-workers from Thailand revealed that all subjects had premature stop codons (*197*). Therefore, restoration of endogenous θ-defensin production does not account for the resistance to HIV-1 infection in these women.

Oral transmission of HIV in adult population is restricted. Examination of differences between adult and infant/fetal oral epithelia indicates that HIV transcytosis can occur through both adult and infant/fetal oral epithelial cells but only HIV passing through fetal cells but not adult cells remains infectious (*198*). Innate effector proteins including HBD2, HBD3 and secretory leukocyte protease inhibitor, predominately expressed in the adult but not infant oral epithelium, contribute to the inactivation of HIV after transcytosis, suggesting that high levels of HBDs may prevent oral transmission of HIV.

Conclusions

Defensins are evolutionally conserved peptides important for host defense. The effect of defensins on viral infection in vitro appears to be defensin, virus and target cell specific. In addition to the direct effect on the virus and target cell, defensins act as immunomodulators that may play an important role in viral transmission and disease progression in vivo. While aberrant defensin expression has been associated with infectious diseases (*199*), metabolic diseases and cancers, the role of defensins in viral transmission and pathogenesis in vivo is still not well established. The complex diversity of defensins among different animal species as well as apparent differences in mechanisms of action remain a challenge in delineating the role of defensins in viral pathogenesis in humans. Further studies focused on the contribution of the structure of defensins to their various effects on viral infections as well as standardization of sample collection methods and assays used to assess their biologic function could reveal some unifying principles and will contribute to their development as novel therapeutics for the prevention of infection.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

References

1. Hackett, C. J. Innate immune activation as a broad-spectrum biodefense strategy: prospects and research challenges. *J. Allergy Clin. Immunol.*. **2003**, *112*, 686–694.
2. Medzhitov, R.; Janeway, C., Jr. Innate immunity. *N. Engl. J. Med.* **2000**, *343*, 338–344.
3. Boman, H. G. Antibacterial peptides: basic facts and emerging concepts. *J. Intern. Med.* **2003**, *254*, 197–215.
4. Ganz, T. Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.*. **2003**, *3*, 710–720.
5. Lehrer, R. I. Primate defensins. *Nat. Rev. Immunol.*. **2004**, *2*, 727–738.
6. Lehrer, R. I.; Lichtenstein, A. K.; Ganz, T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* **1993**, *11*, 105–128.
7. Selsted, M. E.; Ouellette, A. J. Mammalian defensins in the antimicrobial immune response. *Nat. Immunol.* **2005**, *6*, 551–557.
8. Yang, D.; Biragyn, A.; Hoover, D. M.; Lubkowski, J.; Oppenheim, J. J. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu. Rev. Immunol.* **2004**, *22*, 181–215.
9. Rehaume, L. M.; Hancock, R. E. Neutrophil-derived defensins as modulators of innate immune function. *Crit. Rev. Immunol.* **2008**, *28*, 185–200.

10. Yang, D.; Biragyn, A.; Kwak, L. W.; Oppenheim, J. J. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol.* **2002**, *23*, 291–296.
11. Szyk, A.; Wu, Z.; Tucker, K.; Yang, D.; Lu, W.; Lubkowski, J. Crystal structures of human alpha-defensins HNP4, HD5, and HD6. *Protein Sci.* **2006**, *15*, 2749–2760.
12. Pardi, A.; Zhang, X. L.; Selsted, M. E.; Skalicky, J. J.; Yip, P. F. NMR studies of defensin antimicrobial peptides. 2. Three-dimensional structures of rabbit NP-2 and human HNP-1. *Biochemistry* **1992**, *31*, 11357–11364.
13. Hill, C. P.; Yee, J.; Selsted, M. E.; Eisenberg, D. Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. *Science* **1991**, *251*, 1481–1485.
14. Hoover, D. M.; Chertov, O.; Lubkowski, J. The structure of human beta-defensin-1: new insights into structural properties of beta-defensins. *J. Biol. Chem.* **2001**, *276*, 39021–39026.
15. Yamaguchi, Y.; Nagase, T.; Makita, R.; Fukuhara, S.; Tomita, T.; Tominaga, T.; Kurihara, H.; Ouchi, Y. Identification of multiple novel epididymis-specific beta-defensin isoforms in humans and mice. *J. Immunol.* **2002**, *169*, 2516–2523.
16. Pazgier, M.; Hoover, D. M.; Yang, D.; Lu, W.; Lubkowski, J. Human beta-defensins. *Cell Mol. Life Sci.* **2006**, *63*, 1294–1313.
17. Schutte, B. C.; Mitros, J. P.; Bartlett, J. A.; Walters, J. D.; Jia, H. P.; Welsh, M. J.; Casavant, T. L.; McCray, P. B., Jr. Discovery of five conserved beta -defensin gene clusters using a computational search strategy. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2129–2133.
18. Ghosh, D.; Porter, E.; Shen, B.; Lee, S. K.; Wilk, D.; Drazba, J.; Yadav, S. P.; Crabb, J. W.; Ganz, T.; Bevins, C. L. Paneth cell trypsin is the processing enzyme for human defensin-5. *Nat. Immunol.* **2002**, *3*, 583–590.
19. Porter, E.; Yang, H.; Yavagal, S.; Preza, G. C.; Murillo, O.; Lima, H.; Greene, S.; Mahoozi, L.; Klein-Patel, M.; Diamond, G.; et al. Distinct defensin profiles in Neisseria gonorrhoeae and Chlamydia trachomatis urethritis reveal novel epithelial cell-neutrophil interactions. *Infect. Immun.* **2005**, *73*, 4823–4833.
20. Tang, Y. Q.; Yuan, J.; Osapay, G.; Osapay, K.; Tran, D.; Miller, C. J.; Ouellette, A. J.; Selsted, M. E. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* **1999**, *286*, 498–502.
21. Leonova, L.; Kokryakov, V. N.; Aleshina, G.; Hong, T.; Nguyen, T.; Zhao, C.; Waring, A. J.; Lehrer, R. I. Circular minidefensins and posttranslational generation of molecular diversity. *J. Leukocyte Biol.* **2001**, *70*, 461–464.
22. Tran, D.; Tran, P. A.; Tang, Y. Q.; Yuan, J.; Cole, T.; Selsted, M. E. Homodimeric theta-defensins from rhesus macaque leukocytes: isolation, synthesis, antimicrobial activities, and bacterial binding properties of the cyclic peptides. *J. Biol. Chem.* **2002**, *277*, 3079–3084.
23. Tongaonkar, P.; Tran, P.; Roberts, K.; Schaal, J.; Osapay, G.; Tran, D.; Ouellette, A. J.; Selsted, M. E. Rhesus macaque theta-defensin isoforms:

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

expression, antimicrobial activities, and demonstration of a prominent role in neutrophil granule microbicidal activities. *J. Leukocyte Biol.* **2011**, *89*, 283–290.

24. Nguyen, T. X.; Cole, A. M.; Lehrer, R. I. Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides* **2003**, *24*, 1647–1654.
25. Cole, A. M.; Hong, T.; Boo, L. M.; Nguyen, T.; Zhao, C.; Bristol, G.; Zack, J. A.; Waring, A. J.; Yang, O. O.; Lehrer, R. I. Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1813–1818.
26. Maemoto, A.; Qu, X.; Rosengren, K. J.; Tanabe, H.; Henschen-Edman, A.; Craik, D. J.; Ouellette, A. J. Functional analysis of the alpha-defensin disulfide array in mouse cryptdin-4. *J. Biol. Chem.* **2004**, *279*, 44188–44196.
27. Wu, Z.; Hoover, D. M.; Yang, D.; Boulegue, C.; Santamaria, F.; Oppenheim, J. J.; Lubkowski, J.; Lu, W. Engineering disulfide bridges to dissect antimicrobial and Chem.otactic activities of human beta-defensin 3. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 8880–8885.
28. Mandal, M.; Nagaraj, R. Antibacterial activities and conformations of synthetic alpha-defensin HNP-1 and analogs with one, two and three disulfide bridges. *J. Pept. Res.* **2002**, *59*, 95–104.
29. Daher, K. A.; Selsted, M. E.; Lehrer, R. I. Direct inactivation of viruses by human granulocyte defensins. *J. Virol.* **1986**, *60*, 1068–1074.
30. Wang, W.; Cole, A. M.; Hong, T.; Waring, A. J.; Lehrer, R. I. Retrocyclin, an antiretroviral theta-defensin, is a lectin. *J. Immunol.* **2003**, *170*, 4708–4716.
31. Schroeder, B. O.; Wu, Z.; Nuding, S.; Groscurth, S.; Marcinowski, M.; Beisner, J.; Buchner, J.; Schaller, M.; Stange, E. F.; Wehkamp, J. Reduction of disulphide bonds unmasks potent antimicrobial activity of human beta-defensin 1. *Nature* **2011**, *469*, 419–423.
32. Klotman, M. E.; Rapista, A.; Teleshova, N.; Micsenyi, A.; Jarvis, G. A.; Lu, W.; Porter, E.; Chang, T. L. Neisseria gonorrhoeae-Induced Human Defensins 5 and 6 Increase HIV Infectivity: Role in Enhanced Transmission. *J. Immunol.* **2008**, *180*, 6176–6185.
33. Llenado, R. A.; Weeks, C. S.; Cocco, M. J.; Ouellette, A. J. Electropositive charge in alpha-defensin bactericidal activity: functional effects of Lys-for-Arg substitutions vary with the peptide primary structure. *Infect. Immun.* **2009**, *77*, 5035–5043.
34. de Leeuw, E.; Burks, S. R.; Li, X.; Kao, J. P.; Lu, W. Structure-dependent functional properties of human defensin 5. *FEBS Lett.* **2007**, *581*, 515–520.
35. Wei, G.; Pazgier, M.; de Leeuw, E.; Rajabi, M.; Li, J.; Zou, G.; Jung, G.; Yuan, W.; Lu, W. Y.; Lehrer, R. I.; et al. Trp-26 imparts functional versatility to human alpha-defensin HNP1. *J. Biol. Chem.* **2010**, *285*, 16275–16285.
36. Selsted, M. E.; Harwig, S. S.; Ganz, T.; Schilling, J. W.; Lehrer, R. I. Primary structures of three human neutrophil defensins. *J. Clin. Invest.* **1985**, *76*, 1436–1439.
37. Ganz, T.; Selsted, M. E.; Szklarek, D.; Harwig, S. S.; Daher, K.; Bainton, D. F.; Lehrer, R. I. Defensins. Natural peptide antibiotics of human neutrophils. *J. Clin. Invest.* **1985**, *76*, 1427–1435.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

38. Wilde, C. G.; Griffith, J. E.; Marra, M. N.; Snable, J. L.; Scott, R. W. Purification and characterization of human neutrophil peptide 4, a novel member of the defensin family. *J. Biol. Chem.* **1989**, *264*, 11200–11203.
39. Rodriguez-Garcia, M.; Oliva, H.; Climent, N.; Garcia, F.; Gatell, J. M.; Gallart, T. Human immature monocyte-derived dendritic cells produce and secrete alpha-defensins 1-3. *J. Leukocyte Biol.* **2007**, *82*, 1143–1146.
40. Agerberth, B.; Charo, J.; Werr, J.; Olsson, B.; Idali, F.; Lindbom, L.; Kiessling, R.; Jornvall, H.; Wigzell, H.; Gudmundsson, G. H. The human antimicrobial and Chem.otactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* **2000**, *96*, 3086–3093.
41. Ganz, T. Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infect. Immun.* **1987**, *55*, 568–571.
42. Mackewicz, C. E.; Yuan, J.; Tran, P.; Diaz, L.; Mack, E.; Selsted, M. E.; Levy, J. A. alpha-Defensins can have anti-HIV activity but are not CD8 cell anti-HIV factors. *Aids* **2003**, *17*, F23–32.
43. Zaharatos, G. J.; He, T.; Lopez, P.; Yu, W.; Yu, J.; Zhang, L. alpha-Defensins Released Into Stimulated CD8+ T-Cell Supernatants Are Likely Derived From Residual Granulocytes Within the Irradiated Allogeneic Peripheral Blood Mononuclear Cells Used as Feeders. *J. Acquired Immune Defic. Syndr.* **2004**, *36*, 993–1005.
44. Hein, M.; Valore, E. V.; Helmig, R. B.; Uldbjerg, N.; Ganz, T. Antimicrobial factors in the cervical mucus plug. *Am. J. Obstet. Gynecol.* **2002**, *187*, 137–144.
45. Fellermann, K.; Stange, E. F. Defensins -- innate immunity at the epithelial frontier. *Eur. J. Gastroenterol. Hepatol.* **2001**, *13*, 771–776.
46. Cunliffe, R. N. Alpha-defensins in the gastrointestinal tract. *Mol. Immunol.* **2003**, *40*, 463–467.
47. Fan, S. R.; Liu, X. P.; Liao, Q. P. Human defensins and cytokines in vaginal lavage fluid of women with bacterial vaginosis. *Int. J. Gynecol. Obstet.* **2008**.
48. Simhan, H. N.; Anderson, B. L.; Krohn, M. A.; Heine, R. P.; Martinez de Tejada, B.; Landers, D. V.; Hillier, S. L. Host immune consequences of asymptomatic Trichomonas vaginalis infection in pregnancy. *Am. J. Obstet. Gynecol.* **2007**, *196* (59), e51–55.
49. Wiesenfeld, H. C.; Heine, R. P.; Krohn, M. A.; Hillier, S. L.; Amortegui, A. A.; Nicolazzo, M.; Sweet, R. L. Association between elevated neutrophil defensin levels and endometritis. *J. Infect. Dis.* **2002**, *186*, 792–797.
50. Valore, E. V.; Wiley, D. J.; Ganz, T. Reversible deficiency of antimicrobial polypeptides in bacterial vaginosis. *Infect. Immun.* **2006**, *74*, 5693–5702.
51. Jones, D. E.; Bevins, C. L. Paneth cells of the human small intestine express an antimicrobial peptide gene. *J. Biol. Chem.* **1992**, *267*, 23216–23225.
52. Salzman, N. H.; Underwood, M. A.; Bevins, C. L. Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. *Semin Immunol.* **2007**, *19*, 70–83.
53. Tanabe, H.; Ouellette, A. J.; Cocco, M. J.; Robinson, W. E., Jr. Differential effects on human immunodeficiency virus type 1 replication by

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

alpha-defensins with comparable bactericidal activities. *J. Virol.* **2004**, *78*, 11622–11631.

54. Fahlgren, A.; Hammarstrom, S.; Danielsson, A.; Hammarstrom, M. L. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin. Exp. Immunol.* **2003**, *131*, 90–101.
55. Svinarich, D. M.; Wolf, N. A.; Gomez, R.; Gonik, B.; Romero, R. Detection of human defensin 5 in reproductive tissues. *Am. J. Obstet. Gynecol.* **1997**, *176*, 470–475.
56. Quayle, A. J.; Porter, E. M.; Nussbaum, A. A.; Wang, Y. M.; Brabec, C.; Yip, K. P.; Mok, S. C. Gene expression, Immunol.ocalization, and secretion of human defensin-5 in human female reproductive tract. *Am. J. Pathol.* **1998**, *152*, 1247–1258.
57. Radeva, M. Y.; Jahns, F.; Wilhelm, A.; Glei, M.; Settmacher, U.; Greulich, K. O.; Mothes, H. Defensin alpha 6 (DEFA 6) overexpression threshold of over 60 fold can distinguish between adenoma and fully blown colon carcinoma in individual patients. *BMC Cancer* **2010**, *10*, 588.
58. Nam, M. J.; Kee, M. K.; Kuick, R.; Hanash, S. M. Identification of defensin alpha6 as a potential biomarker in colon adenocarcinoma. *J. Biol. Chem.* **2005**, *280*, 8260–8265.
59. Duits, L. A.; Ravensbergen, B.; Rademaker, M.; Hiemstra, P. S.; Nibbering, P. H. Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. *Immunology* **2002**, *106*, 517–525.
60. Ryan, L. K.; Dai, J.; Yin, Z.; Megjugorac, N.; Uhlhorn, V.; Yim, S.; Schwartz, K. D.; Abrahams, J. M.; Diamond, G.; Fitzgerald-Bocarsly, P. Modulation of human {beta}-defensin-1 (hBD-1) in plasmacytoid dendritic cells (PDC), monocytes, and epithelial cells by influenza virus, Herpes simplex virus, and Sendai virus and its possible role in innate immunity. *J. Leukocyte Biol.* **2011**.
61. Ryan, L. K.; Diamond, G.; Amrute, S.; Feng, Z.; Weinberg, A.; Fitzgerald-Bocarsly, P. Detection of HBD1 peptide in peripheral blood mononuclear cell subpopulations by intracellular flow cytometry. *Peptides* **2003**, *24*, 1785–1794.
62. Fang, X. M.; Shu, Q.; Chen, Q. X.; Book, M.; Sahl, H. G.; Hoeft, A.; Stuber, F. Differential expression of alpha- and beta-defensins in human peripheral blood. *Eur. J. Clin. Invest.* **2003**, *33*, 82–87.
63. Proud, D.; Sanders, S. P.; Wiehler, S. Human rhinovirus infection induces airway epithelial cell production of human beta-defensin 2 both in vitro and in vivo. *J. Immunol.* **2004**, *172*, 4637–4645.
64. Duits, L. A.; Nibbering, P. H.; van Strijen, E.; Vos, J. B.; Mannesse-Lazeroms, S. P.; van Sterkenburg, M. A.; Hiemstra, P. S. Rhinovirus increases human beta-defensin-2 and -3 mRNA expression in cultured bronchial epithelial cells. *FEMS Immunol. Med. Microbiol.* **2003**, *38*, 59–64.
65. Yang, D.; Chertov, O.; Oppenheim, J. J. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and

activities of human defensins and cathelicidin (LL-37). *J. Leukocyte Biol.* **2001**, *69*, 691–697.

66. Sorensen, O. E.; Thapa, D. R.; Rosenthal, A.; Liu, L.; Roberts, A. A.; Ganz, T. Differential regulation of beta-defensin expression in human skin by microbial stimuli. *J. Immunol.* **2005**, *174*, 4870–4879.
67. Harder, J.; Bartels, J.; Christophers, E.; Schroder, J. M. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J. Biol. Chem.* **2001**, *276*, 5707–5713.
68. Garcia, J. R.; Jaumann, F.; Schulz, S.; Krause, A.; Rodriguez-Jimenez, J.; Forssmann, U.; Adermann, K.; Kluver, E.; Vogelmeier, C.; Becker, D.; et al. Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage Chem.oattraction. *Cell Tissue Res.* **2001**, *306*, 257–264.
69. Armogida, S. A.; Yannaras, N. M.; Melton, A. L.; Srivastava, M. D. Identification and quantification of innate immune system mediators in human breast milk. *Allergy Asthma Proc.* **2004**, *25*, 297–304.
70. Jia, H. P.; Starner, T.; Ackermann, M.; Kirby, P.; Tack, B. F.; McCray, P. B., Jr. Abundant human beta-defensin-1 expression in milk and mammary gland epithelium. *J. Pediatr.* **2001**, *138*, 109–112.
71. Coffelt, S. B.; Scandurro, A. B. Tumors sound the alarmin(s). *Cancer Res.* **2008**, *68*, 6482–6485.
72. Liu, H. Y.; Collins, Q. F.; Moukdar, F.; Zhuo, D.; Han, J.; Hong, T.; Collins, S.; Cao, W. Suppression of hepatic glucose production by human neutrophil alpha-defensins through a signaling pathway distinct from insulin. *J. Biol. Chem.* **2008**, *283*, 12056–12063.
73. Joseph, G.; Tarnow, L.; Astrup, A. S.; Hansen, T. K.; Parving, H. H.; Flyvbjerg, A.; Frystyk, J. Plasma alpha-defensin is associated with cardiovascular morbidity and mortality in type 1 diabetic patients. *J. Clin Endocrinol. Metab.* **2008**, *93*, 1470–1475.
74. Saraheimo, M.; Forsblom, C.; Pettersson-Fernholm, K.; Flyvbjerg, A.; Groop, P. H.; Frystyk, J. Increased levels of alpha-defensin (-1, -2 and -3) in type 1 diabetic patients with nephropathy. *Nephrol. Dial Transplant* **2008**, *23*, 914–918.
75. Kruse, T.; Kristensen, H. H. Using antimicrobial host defense peptides as anti-infective and immunomodulatory agents. *Expert Rev. Anti Infect. Ther.* **2008**, *6*, 887–895.
76. Yang, D.; Chen, Q.; Chertov, O.; Oppenheim, J. J. Human neutrophil defensins selectively Chem.oattract naive T and immature dendritic cells. *J. Leukocyte Biol.* **2000**, *68*, 9–14.
77. Chertov, O.; Michiel, D. F.; Xu, L.; Wang, J. M.; Tani, K.; Murphy, W. J.; Longo, D. L.; Taub, D. D.; Oppenheim, J. J. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell Chem.oattractant proteins released from interleukin-8-stimulated neutrophils. *J. Biol. Chem.* **1996**, *271*, 2935–2940.
78. Tecle, T.; White, M. R.; Gantz, D.; Crouch, E. C.; Hartshorn, K. L. Human neutrophil defensins increase neutrophil uptake of influenza A virus and

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

bacteria and modify virus-induced respiratory burst responses. *J. Immunol.* **2007**, *178*, 8046–8052.

79. Shi, J.; Aono, S.; Lu, W.; Ouellette, A. J.; Hu, X.; Ji, Y.; Wang, L.; Lenz, S.; van Ginkel, F. W.; Liles, M.; et al. A novel role for defensins in intestinal homeostasis: regulation of IL-1beta secretion. *J. Immunol.* **2007**, *179*, 1245–1253.
80. Van Wetering, S.; Mannesse-Lazeroms, S. P.; Van Sterkenburg, M. A.; Daha, M. R.; Dijkman, J. H.; Hiemstra, P. S. Effect of defensins on interleukin-8 synthesis in airway epithelial cells. *Am. J. Physiol.* **1997**, *272*, L888–896.
81. Guo, C. J.; Tan, N.; Song, L.; Douglas, S. D.; Ho, W. Z. Alpha-defensins inhibit HIV infection of macrophages through upregulation of CC-Chem.okines. *Aids* **2004**, *18*, 1217–1218.
82. Liu, C. Y.; Lin, H. C.; Yu, C. T.; Lin, S. M.; Lee, K. Y.; Chen, H. C.; Chou, C. L.; Huang, C. D.; Chou, P. C.; Liu, W. T.; et al. The concentration-dependent Chem.okine release and pro-apoptotic effects of neutrophil-derived alpha-defensin-1 on human bronchial and alveolar epithelial cells. *Life Sci* **2007**, *80*, 749–758.
83. Yang, D.; Chertov, O.; Bykovskaia, S. N.; Chen, Q.; Buffo, M. J.; Shogan, J.; Anderson, M.; Schroder, J. M.; Wang, J. M.; Howard, O. M.; et al. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **1999**, *286*, 525–528.
84. Zlotnik, A.; Yoshie, O. Chem.okines: a new classification system and their role in immunity. *Immunity* **2000**, *12*, 121–127.
85. Boniotto, M.; Jordan, W. J.; Eskdale, J.; Tossi, A.; Antcheva, N.; Crovella, S.; Connell, N. D.; Gallagher, G. Human beta-defensin 2 induces a vigorous cytokine response in peripheral blood mononuclear cells. *Antimicrob. Agents Chem.other* **2006**, *50*, 1433–1441.
86. Niyonsaba, F.; Hirata, M.; Ogawa, H.; Nagaoka, I. Epithelial cell-derived antibacterial peptides human beta-defensins and cathelicidin: multifunctional activities on mast cells. *Curr. Drug Targets Inflamm. Allergy* **2003**, *2*, 224–231.
87. Biragyn, A.; Ruffini, P. A.; Leifer, C. A.; Klyushnenkova, E.; Shakhov, A.; Chertov, O.; Shirakawa, A. K.; Farber, J. M.; Segal, D. M.; Oppenheim, J. J.; et al. Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* **2002**, *298*, 1025–1029.
88. Funderburg, N.; Lederman, M. M.; Feng, Z.; Drage, M. G.; Jadlowsky, J.; Harding, C. V.; Weinberg, A.; Sieg, S. F. Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 18631–18635.
89. Jin, G.; Kawsar, H. I.; Hirsch, S. A.; Zeng, C.; Jia, X.; Feng, Z.; Ghosh, S. K.; Zheng, Q. Y.; Zhou, A.; McIntyre, T. M.; et al. An antimicrobial peptide regulates tumor-associated macrophage trafficking via the Chem.okine receptor CCR2, a model for tumorigenesis. *PLoS One* **2010**, *5*, e10993.
90. Feng, Z.; Dubyak, G. R.; Lederman, M. M.; Weinberg, A. Cutting edge: human beta defensin 3--a novel antagonist of the HIV-1 coreceptor CXCR4. *J. Immunol.* **2006**, *177*, 782–786.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

91. Seidel, A.; Ye, Y.; de Armas, L. R.; Soto, M.; Yarosh, W.; Marcsisin, R. A.; Tran, D.; Selsted, M. E.; Camerini, D. Cyclic and acyclic defensins inhibit human immunodeficiency virus type-1 replication by different mechanisms. *PLoS One* **2010**, *5*, e9737.
92. Quinones-Mateu, M. E.; Lederman, M. M.; Feng, Z.; Chakraborty, B.; Weber, J.; Rangel, H. R.; Marotta, M. L.; Mirza, M.; Jiang, B.; Kiser, P.; et al. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. *Aids* **2003**, *17*, F39–48.
93. Rohrl, J.; Yang, D.; Oppenheim, J. J.; Hehlgans, T. Human beta-defensin 2 and 3 and their mouse orthologs induce Chem.otaxis through interaction with CCR2. *J. Immunol.* **2010**, *184*, 6688–6694.
94. Nassar, T.; Akkawi, S.; Bar-Shavit, R.; Haj-Yehia, A.; Bdeir, K.; Al-Mehdi, A. B.; Tarshis, M.; Higazi, A. A. Human alpha-defensin regulates smooth muscle cell contraction: a role for low-density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *Blood* **2002**, *100*, 4026–4032.
95. Higazi, A. A.; Nassar, T.; Ganz, T.; Rader, D. J.; Udassin, R.; Bdeir, K.; Hiss, E.; Sachais, B. S.; Williams, K. J.; Leitersdorf, E.; et al. The alpha-defensins stimulate proteoglycan-dependent catabolism of low-density lipoprotein by vascular cells: a new class of inflammatory apolipoprotein and a possible contributor to atherogenesis. *Blood* **2000**, *96*, 1393–1398.
96. Higazi, A. A.; Ganz, T.; Kariko, K.; Cines, D. B. Defensin modulates tissue-type plasminogen activator and plasminogen binding to fibrin and endothelial cells. *J. Biol. Chem.* **1996**, *271*, 17650–17655.
97. Charp, P. A.; Rice, W. G.; Raynor, R. L.; Reimund, E.; Kinkade, J. M., Jr.; Ganz, T.; Selsted, M. E.; Lehrer, R. I.; Kuo, J. F. Inhibition of protein kinase C by defensins, antibiotic peptides from human neutrophils. *Biochem. Pharmacol.* **1988**, *37*, 951–956.
98. Chang, T. L.; Vargas, J., Jr.; DelPortillo, A.; Klotman, M. E. Dual role of alpha-defensin-1 in anti-HIV-1 innate immunity. *J. Clin. Invest.* **2005**, *115*, 765–773.
99. Salvatore, M.; Garcia-Sastre, A.; Ruchala, P.; Lehrer, R. I.; Chang, T.; Klotman, M. E. alpha-Defensin inhibits influenza virus replication by cell-mediated mechanism(s). *J. Infect. Dis* **2007**, *196*, 835–843.
100. van den Berg, R. H.; Faber-Krol, M. C.; van Wetering, S.; Hiemstra, P. S.; Daha, M. R. Inhibition of activation of the classical pathway of complement by human neutrophil defensins. *Blood* **1998**, *92*, 3898–3903.
101. Groeneveld, T. W.; Ramwadhdoebe, T. H.; Trouw, L. A.; van den Ham, D. L.; van der Borden, V.; Drijfhout, J. W.; Hiemstra, P. S.; Daha, M. R.; Roos, A. Human neutrophil peptide-1 inhibits both the classical and the lectin pathway of complement activation. *Mol. Immunol.* **2007**, *44*, 3608–3614.
102. Cowland, J. B.; Borregaard, N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *J. Leukocyte Biol.* **1999**, *66*, 989–995.
103. Tsutsumi-Ishii, Y.; Hasebe, T.; Nagaoka, I. Role of CCAAT/enhancer-binding protein site in transcription of human neutrophil peptide-1 and -3 defensin genes. *J. Immunol.* **2000**, *164*, 3264–3273.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

104. Joiner, K. A.; Ganz, T.; Albert, J.; Rotrosen, D. The opsonizing ligand on Salmonella typhimurium influences incorporation of specific, but not azurophil, granule constituents into neutrophil phagosomes. *J. Cell Biol.* **1989**, *109*, 2771–2782.

105. Tanaka, S.; Edberg, J. C.; Chatham, W.; Fassina, G.; Kimberly, R. P. Fc gamma RIIIb allele-sensitive release of alpha-defensins: anti-neutrophil cytoplasmic antibody-induced release of Chem.otaxins. *J. Immunol.* **2003**, *171*, 6090–6096.

106. Chalifour, A.; Jeannin, P.; Gauchat, J. F.; Blaecke, A.; Malissard, M.; N'Guyen, T.; Thieblemont, N.; Delneste, Y. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production. *Blood* **2004**, *104*, 1778–1783.

107. Jan, M. S.; Huang, Y. H.; Shieh, B.; Teng, R. H.; Yan, Y. P.; Lee, Y. T.; Liao, K. K.; Li, C. CC Chem.okines Induce Neutrophils to Chem.otaxis, Degranulation, and alpha-Defensin Release. *J. Acquired Immune Defic. Syndr.* **2006**, *41*, 6–16.

108. Driss, V.; Legrand, F.; Hermann, E.; Loiseau, S.; Guerardel, Y.; Kremer, L.; Adam, E.; Woerly, G.; Dombrowicz, D.; Capron, M. TLR2-dependent eosinophil interactions with mycobacteria : role of {alpha}-defensins. *Blood* **2008**.

109. Cunliffe, R. N.; Rose, F. R.; Keyte, J.; Abberley, L.; Chan, W. C.; Mahida, Y. R. Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. *Gut* **2001**, *48*, 176–185.

110. George, M. D.; Wehkamp, J.; Kays, R. J.; Leutenegger, C. M.; Sabir, S.; Grishina, I.; Dandekar, S.; Bevins, C. L. In vivo gene expression profiling of human intestinal epithelial cells: analysis by laser microdissection of formalin fixed tissues. *BMC Genomics* **2008**, *9*, 209.

111. Wehkamp, J.; Harder, J.; Weichenthal, M.; Schwab, M.; Schaffeler, E.; Schlee, M.; Herrlinger, K. R.; Stallmach, A.; Noack, F.; Fritz, P.; et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* **2004**, *53*, 1658–1664.

112. Wehkamp, J.; Wang, G.; Kubler, I.; Nuding, S.; Gregorieff, A.; Schnabel, A.; Kays, R. J.; Fellermann, K.; Burk, O.; Schwab, M.; et al. The Paneth cell alpha-defensin deficiency of ileal Crohn's disease is linked to Wnt/Tcf-4. *J. Immunol.* **2007**, *179*, 3109–3118.

113. Hertz, C. J.; Wu, Q.; Porter, E. M.; Zhang, Y. J.; Weismuller, K. H.; Godowski, P. J.; Ganz, T.; Randell, S. H.; Modlin, R. L. Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J. Immunol.* **2003**, *171*, 6820–6826.

114. Vora, P.; Youdim, A.; Thomas, L. S.; Fukata, M.; Tesfay, S. Y.; Lukasek, K.; Michelsen, K. S.; Wada, A.; Hirayama, T.; Arditi, M.; et al. Beta-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *J. Immunol.* **2004**, *173*, 5398–5405.

115. Uehara, A.; Fujimoto, Y.; Fukase, K.; Takada, H. Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce

anti-microbial peptides, but not proinflammatory cytokines. *Mol. Immunol.* **2007**, *44*, 3100–3111.

116. Nagy, I.; Pivarcsi, A.; Koreck, A.; Szell, M.; Urban, E.; Kemeny, L. Distinct strains of Propionibacterium acnes induce selective human beta-defensin-2 and interleukin-8 expression in human keratinocytes through toll-like receptors. *J. Invest. Dermatol.* **2005**, *124*, 931–938.
117. Pivarcsi, A.; Nagy, I.; Koreck, A.; Kis, K.; Kenderessy-Szabo, A.; Szell, M.; Dobozy, A.; Kemeny, L. Microbial compounds induce the expression of pro-inflammatory cytokines, Chem.okines and human beta-defensin-2 in vaginal epithelial cells. *Microbes Infect.* **2005**, *7*, 1117–1127.
118. Schaefer, T. M.; Fahey, J. V.; Wright, J. A.; Wira, C. R. Innate immunity in the human female reproductive tract: antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C). *J. Immunol.* **2005**, *174*, 992–1002.
119. Uehara, A.; Takada, H. Synergism between TLRs and NOD1/2 in oral epithelial cells. *J. Dent. Res.* **2008**, *87*, 682–686.
120. Huang, F.; Kao, C. Y.; Wachi, S.; Thai, P.; Ryu, J.; Wu, R. Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-kappaB activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. *J. Immunol.* **2007**, *179*, 6504–6513.
121. Jang, B. C.; Lim, K. J.; Suh, M. H.; Park, J. G.; Suh, S. I. Dexamethasone suppresses interleukin-1beta-induced human beta-defensin 2 mRNA expression: involvement of p38 MAPK, JNK, MKP-1, and NF-kappaB transcriptional factor in A549 cells. *FEMS Immunol. Med. Microbiol.* **2007**, *51*, 171–184.
122. Kao, C. Y.; Kim, C.; Huang, F.; Wu, R. Requirements for two proximal NF-kappaB binding sites and IkappaB-zeta in IL-17A-induced human beta-defensin 2 expression by conducting airway epithelium. *J. Biol. Chem.* **2008**, *283*, 15309–15318.
123. Pernet, I.; Reymermier, C.; Guezennec, A.; Branka, J. E.; Guesnet, J.; Perrier, E.; Dezutter-Dambuyant, C.; Schmitt, D.; Viac, J. Calcium triggers beta-defensin (hBD-2 and hBD-3) and Chem.okine macrophage inflammatory protein-3 alpha (MIP-3alpha/CCL20) expression in monolayers of activated human keratinocytes. *Exp. Dermatol.* **2003**, *12*, 755–760.
124. Nittayananta, W.; Hladik, F.; Klausner, M.; Harb, S.; Dale, B. A.; Coombs, R. W. HIV type 1 fails to trigger innate immune factor synthesis in differentiated oral epithelium. *AIDS Res. Hum. Retroviruses* **2009**, *25*, 1013–1021.
125. Chong, K. T.; Thangavel, R. R.; Tang, X. Enhanced expression of murine beta-defensins (MBD-1, -2,- 3, and -4) in upper and lower airway mucosa of influenza virus infected mice. *Virology* **2008**, *380*, 136–143.
126. Kota, S.; Sabbah, A.; Chang, T. H.; Harnack, R.; Xiang, Y.; Meng, X.; Bose, S. Role of human beta-defensin-2 during tumor necrosis factor-alpha/NF-kappaB-mediated innate antiviral response against human respiratory syncytial virus. *J. Biol. Chem.* **2008**, *283*, 22417–22429.
127. Van Wetering, S.; Mannesse-Lazeroms, S. P.; Dijkman, J. H.; Hiemstra, P. S. Effect of neutrophil serine proteinases and defensins on lung epithelial cells:

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

modulation of cytotoxicity and IL-8 production. *J. Leukocyte Biol.* **1997**, *62*, 217–226.

128. Nakashima, H.; Yamamoto, N.; Masuda, M.; Fujii, N. Defensins inhibit HIV replication in vitro. *Aids* **1993**, *7*, 1129.
129. Furci, L.; Sironi, F.; Tolazzi, M.; Vassena, L.; Lusso, P. Alpha-defensins block the early steps of HIV-1 infection: interference with the binding of gp120 to CD4. *Blood* **2007**, *109*, 2928–2935.
130. Zhang, L.; Yu, W.; He, T.; Yu, J.; Caffrey, R. E.; Dalmasso, E. A.; Fu, S.; Pham, T.; Mei, J.; Ho, J. J.; et al. Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* **2002**, *298*, 995–1000.
131. Chang, T. L.; Francois, F.; Mosoian, A.; Klotman, M. E. CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from alpha-defensin-1 HIV inhibition. *J. Virol.* **2003**, *77*, 6777–6784.
132. Wu, Z.; Cocchi, F.; Gentles, D.; Ericksen, B.; Lubkowski, J.; Devico, A.; Lehrer, R. I.; Lu, W. Human neutrophil alpha-defensin 4 inhibits HIV-1 infection in vitro. *FEBS Lett.* **2005**, *579*, 162–166.
133. Territo, M. C.; Ganz, T.; Selsted, M. E.; Lehrer, R. Monocyte-Chem.otactic activity of defensins from human neutrophils. *J. Clin. Invest.* **1989**, *84*, 2017–2020.
134. Wang, W.; Owen, S. M.; Rudolph, D. L.; Cole, A. M.; Hong, T.; Waring, A. J.; Lal, R. B.; Lehrer, R. I. Activity of alpha- and theta-defensins against primary isolates of HIV-1. *J. Immunol.* **2004**, *173*, 515–520.
135. Rapista, A.; Ding, J.; Benito, B.; Lo, Y. T.; Neiditch, M. B.; Lu, W.; Chang, T. L. Human defensins 5 and 6 enhance HIV-1 infectivity through promoting HIV attachment. *Retrovirology* **2011**, *8*, 45.
136. Ding, J.; Rapista, A.; Teleshova, N.; Lu, W.; Klotman, M. E.; Chang, T. L. Mucosal human defensins 5 and 6 antagonize the anti-HIV activity of candidate polyanion microbicides. *J. Innate Immun.* **2011**, *3*, 208–212.
137. Sun, L.; Finnegan, C. M.; Kish-Catalone, T.; Blumenthal, R.; Garzino-Demo, P.; La Terra Maggiore, G. M.; Berrone, S.; Kleinman, C.; Wu, Z.; Abdelwahab, S.; et al. Human {beta}-Defensins Suppress Human Immunodeficiency Virus Infection: Potential Role in Mucosal Protection. *J. Virol.* **2005**, *79*, 14318–14329.
138. Lafferty, M. K.; Sun, L.; DeMasi, L.; Lu, W.; Garzino-Demo, A. CCR6 ligands inhibit HIV by inducing APOBEC3G. *Blood* **2010**, *115*, 1564–1571.
139. Munk, C.; Wei, G.; Yang, O. O.; Waring, A. J.; Wang, W.; Hong, T.; Lehrer, R. I.; Landau, N. R.; Cole, A. M. The theta-defensin, retrocyclin, inhibits HIV-1 entry. *AIDS Res Hum Retroviruses* **2003**, *19*, 875–881.
140. Gallo, S. A.; Wang, W.; Rawat, S. S.; Jung, G.; Waring, A. J.; Cole, A. M.; Lu, H.; Yan, X.; Daly, N. L.; Craik, D. J.; et al. Theta-defensins prevent HIV-1 Env-mediated fusion by binding gp41 and blocking 6-helix bundle formation. *J. Biol. Chem.* **2006**, *281*, 18787–18792.
141. Owen, S. M.; Rudolph, D. L.; Wang, W.; Cole, A. M.; Waring, A. J.; Lal, R. B.; Lehrer, R. I. RC-101, a retrocyclin-1 analogue with enhanced activity

against primary HIV type 1 isolates. *AIDS Res. Hum. Retroviruses* **2004**, *20*, 1157–1165.

142. Sinha, S.; Cheshenko, N.; Lehrer, R. I.; Herold, B. C. NP-1, a rabbit alpha-defensin, prevents the entry and intercellular spread of herpes simplex virus type 2. *Antimicrob. Agents Chemother* **.2003**, *47*, 494–500.
143. Yasin, B.; Wang, W.; Pang, M.; Cheshenko, N.; Hong, T.; Waring, A. J.; Herold, B. C.; Wagar, E. A.; Lehrer, R. I. Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *J. Virol.* **2004**, *78*, 5147–5156.
144. Hazrati, E.; Galen, B.; Lu, W.; Wang, W.; Ouyang, Y.; Keller, M. J.; Lehrer, R. I.; Herold, B. C. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. *J. Immunol.* **2006**, *177*, 8658–8666.
145. Hartshorn, K. L.; White, M. R.; Tecle, T.; Holmskov, U.; Crouch, E. C. Innate defense against influenza A virus: activity of human neutrophil defensins and interactions of defensins with surfactant protein D. *J. Immunol.* **2006**, *176*, 6962–6972.
146. Leikina, E.; Delanoe-Ayari, H.; Melikov, K.; Cho, M. S.; Chen, A.; Waring, A. J.; Wang, W.; Xie, Y.; Loo, J. A.; Lehrer, R. I.; et al. Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. *Nat. Immunol.* **2005**, *6*, 995–1001.
147. Doss, M.; White, M. R.; Tecle, T.; Gantz, D.; Crouch, E. C.; Jung, G.; Ruchala, P.; Waring, A. J.; Lehrer, R. I.; Hartshorn, K. L. Interactions of alpha-, beta-, and theta-defensins with influenza A virus and surfactant protein D. *J. Immunol.* **2009**, *182*, 7878–7887.
148. Grubor, B.; Gallup, J. M.; Meyerholz, D. K.; Crouch, E. C.; Evans, R. B.; Brogden, K. A.; Lehmkuhl, H. D.; Ackermann, M. R. Enhanced surfactant protein and defensin mRNA levels and reduced viral replication during parainfluenza virus type 3 pneumonia in neonatal lambs. *Clin. Diagn. Lab Immunol.* **2004**, *11*, 599–607.
149. Meyerholz, D. K.; Grubor, B.; Gallup, J. M.; Lehmkuhl, H. D.; Anderson, R. D.; Lazic, T.; Ackermann, M. R. Adenovirus-mediated gene therapy enhances parainfluenza virus 3 infection in neonatal lambs. *J. Clin. Microbiol.* **2004**, *42*, 4780–4787.
150. Peiris, J. S.; Yuen, K. Y.; Osterhaus, A. D.; Stohr, K. The severe acute respiratory syndrome. *N. Engl. J. Med.* **2003**, *349*, 2431–2441.
151. Wohlford-Lenane, C. L.; Meyerholz, D. K.; Perlman, S.; Zhou, H.; Tran, D.; Selsted, M. E.; McCray, P. B., Jr. Rhesus theta-defensin prevents death in a mouse model of severe acute respiratory syndrome coronavirus pulmonary disease. *J. Virol.* **2009**, *83*, 11385–11390.
152. Howell, M. D.; Jones, J. F.; Kisich, K. O.; Streib, J. E.; Gallo, R. L.; Leung, D. Y. Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J. Immunol.* **2004**, *172*, 1763–1767.
153. Howell, M. D.; Streib, J. E.; Leung, D. Y. Antiviral activity of human beta-defensin 3 against vaccinia virus. *J. Allergy Clin. Immunol.* **2007**, *119*, 1022–1025.
154. Dugan, A. S.; Maginnis, M. S.; Jordan, J. A.; Gasparovic, M. L.; Manley, K.; Page, R.; Williams, G.; Porter, E.; O'Hara, B. A.; Atwood, W. J. Human

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

alpha-defensins inhibit BK virus infection by aggregating virions and blocking binding to host cells. *J. Biol. Chem.* **2008**, *283*, 31125–31132.

155. Bastian, A.; Schafer, H. Human alpha-defensin 1 (HNP-1) inhibits adenoviral infection in vitro. *Regul. Pept.* **2001**, *101*, 157–161.
156. Harvey, S. A.; Romanowski, E. G.; Yates, K. A.; Gordon, Y. J. Adenovirus-directed ocular innate immunity: the role of conjunctival defensin-like Chem.okines (IP-10, I-TAC) and phagocytic human defensin-alpha. *Invest. Ophthalmol. Vis. Sci.* **2005**, *46*, 3657–3665.
157. Gropp, R.; Frye, M.; Wagner, T. O.; Bargon, J. Epithelial defensins impair adenoviral infection: implication for adenovirus-mediated gene therapy. *Hum. Gene Ther.* **1999**, *10*, 957–964.
158. Smith, J. G.; Nemerow, G. R. Mechanism of adenovirus neutralization by Human alpha-defensins. *Cell Host Microbe* **2008**, *3*, 11–19.
159. Nguyen, E. K.; Nemerow, G. R.; Smith, J. G. Direct evidence from single-cell analysis that human {alpha}-defensins block adenovirus uncoating to neutralize infection. *J. Virol.* **2010**, *84*, 4041–4049.
160. Smith, J. G.; Silvestry, M.; Lindert, S.; Lu, W.; Nemerow, G. R.; Stewart, P. L. Insight into the mechanisms of adenovirus capsid disassembly from studies of defensin neutralization. *PLoS Pathog.* **2010**, *6*, e1000959.
161. Buck, C. B.; Day, P. M.; Thompson, C. D.; Lubkowski, J.; Lu, W.; Lowy, D. R.; Schiller, J. T. Human {alpha}-defensins block papillomavirus infection. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 1516–1521.
162. Yang, D.; de la Rosa, G.; Tewary, P.; Oppenheim, J. J. Alarmins link neutrophils and dendritic cells. *Trends Immunol.* **2009**, *30*, 531–537.
163. Hoang, L. T.; Lynn, D. J.; Henn, M.; Birren, B. W.; Lennon, N. J.; Le, P. T.; Duong, K. T.; Nguyen, T. T.; Mai, L. N.; Farrar, J. J.; et al. The early whole-blood transcriptional signature of dengue virus and features associated with progression to dengue shock syndrome in Vietnamese children and young adults. *J. Virol.* **2010**, *84*, 12982–12994.
164. Kreuter, A.; Skrygan, M.; Gambichler, T.; Brockmeyer, N. H.; Stucker, M.; Herzler, C.; Potthoff, A.; Altmeyer, P.; Pfister, H.; Wieland, U. Human papillomavirus-associated induction of human beta-defensins in anal intraepithelial neoplasia. *Br. J. Dermatol.* **2009**, *160*, 1197–1205.
165. Panyutich, A. V.; Szold, O.; Poon, P. H.; Tseng, Y.; Ganz, T. Identification of defensin binding to C1 complement. *FEBS Lett.* **1994**, *356*, 169–173.
166. Panyutich, A.; Ganz, T. Activated alpha 2-macroglobulin is a principal defensin-binding protein. *Am. J. Respir Cell Mol Biol.* **1991**, *5*, 101–106.
167. Mukae, H.; Iiboshi, H.; Nakazato, M.; Hiratsuka, T.; Tokojima, M.; Abe, K.; Ashitani, J.; Kadota, J.; Matsukura, S.; Kohno, S. Raised plasma concentrations of alpha-defensins in patients with idiopathic pulmonary fibrosis. *Thorax* **2002**, *57*, 623–628.
168. Levinson, P.; Kaul, R.; Kimani, J.; Ngugi, E.; Moses, S.; MacDonald, K. S.; Broliden, K.; Hirbod, T. Levels of innate immune factors in genital fluids: association of alpha defensins and LL-37 with genital infections and increased HIV acquisition. *AIDS* **2009**, *23*, 309–317.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

169. Ihi, T.; Nakazato, M.; Mukae, H.; Matsukura, S. Elevated concentrations of human neutrophil peptides in plasma, blood, and body fluids from patients with infections. *Clin. Infect. Dis* **1997**, *25*, 1134–1140.

170. Panyutich, A. V.; Panyutich, E. A.; Krapivin, V. A.; Baturevich, E. A.; Ganz, T. Plasma defensin concentrations are elevated in patients with septicemia or bacterial meningitis. *J. Lab Clin. Med.* **1993**, *122*, 202–207.

171. Gardner, M. S.; Rowland, M. D.; Siu, A. Y.; Bundy, J. L.; Wagener, D. K.; Stephenson, J. L. Comprehensive defensin assay for saliva. *Anal. Chem.* **2009**, *81*, 557–566.

172. Ghosh, S. K.; Gerken, T. A.; Schneider, K. M.; Feng, Z.; McCormick, T. S.; Weinberg, A. Quantification of human beta-defensin-2 and -3 in body fluids: application for studies of innate immunity. *Clin. Chem.* **2007**, *53*, 757–765.

173. Trabattoni, D.; Caputo, S. L.; Maffeis, G.; Vichi, F.; Biasin, M.; Pierotti, P.; Fasano, F.; Saresella, M.; Franchini, M.; Ferrante, P.; et al. Human alpha Defensin in HIV-Exposed But Uninfected Individuals. *J. Acquired Immune Defic. Syndr.* **2004**, *35*, 455–463.

174. D'Agostino, C.; Lichtner, M.; Mastroianni, C. M.; Ceccarelli, G.; Iannetta, M.; Antonucci, S.; Vullo, V.; Massetti, A. P. In vivo release of alpha-defensins in plasma, neutrophils and CD8 T-lymphocytes of patients with HIV infection. *Curr. HIV Res.* **2009**, *7*, 650–655.

175. Rodriguez-Garcia, M.; Climent, N.; Oliva, H.; Casanova, V.; Franco, R.; Leon, A.; Gatell, J. M.; Garcia, F.; Gallart, T. Increased alpha-defensins 1-3 production by dendritic cells in HIV-infected individuals is associated with slower disease progression. *PloS One* **2010**, *5*, e9436.

176. Folkvord, J. M.; Armon, C.; Connick, E. Lymphoid follicles are sites of heightened human immunodeficiency virus type 1 (HIV-1) replication and reduced antiretroviral effector mechanisms. *AIDS Res. Hum. Retroviruses* **2005**, *21*, 363–370.

177. Kuhn, L.; Trabattoni, D.; Kankasa, C.; Semrau, K.; Kasonde, P.; Lissoni, F.; Sinkala, M.; Ghosh, M.; Vwalika, C.; Aldrovandi, G. M.; et al. Alpha-defensins in the prevention of HIV transmission among breastfed infants. *J. Acquired Immune Defic. Syndr.* **2005**, *39*, 138–142.

178. Bosire, R.; John-Stewart, G. C.; Mabuka, J. M.; Wariua, G.; Gichuhi, C.; Wamalwa, D.; Ruzinski, J.; Goodman, R.; Lohman, B.; Mbori-Ngacha, D. A.; et al. Breast milk alpha-defensins are associated with HIV type 1 RNA and CC Chem.okines in breast milk but not vertical HIV type 1 transmission. *AIDS Res. Hum. Retroviruses* **2007**, *23*, 198–203.

179. Venkataraman, N.; Cole, A. L.; Svoboda, P.; Pohl, J.; Cole, A. M. Cationic polypeptides are required for anti-HIV-1 activity of human vaginal fluid. *J. Immunol.* **2005**, *175*, 7560–7567.

180. Galvin, S. R.; Cohen, M. S. The role of sexually transmitted diseases in HIV transmission. *Nat. Rev. Immunol..* **2004**, *2*, 33–42.

181. Plummer, F. A. Heterosexual transmission of human immunodeficiency virus type 1 (HIV): interactions of conventional sexually transmitted diseases, hormonal contraception and HIV-1. *AIDS Res. Hum. Retroviruses* **1998**, *14* (Suppl 1), S5–10.

182. Cohen, M. S.; Hoffman, I. F.; Royce, R. A.; Kazembe, P.; Dyer, J. R.; Daly, C. C.; Zimba, D.; Vernazza, P. L.; Maida, M.; Fiscus, S. A.; et al. Reduction of concentration of HIV-1 in semen after treatment of urethritis: implications for prevention of sexual transmission of HIV-1. AIDSCAP Malawi Research Group. *Lancet* **1997**, *349*, 1868–1873.

183. Chesson, H. W.; Pinkerton, S. D. Sexually transmitted diseases and the increased risk for HIV transmission: implications for cost-effectiveness analyses of sexually transmitted disease prevention interventions. *J. Acquired Immune Defic. Syndr.* **2000**, *24*, 48–56.

184. Mabey, D. Interactions between HIV infection and other sexually transmitted diseases. *Trop. Med. Int. Health* **2000**, *5*, A32–36.

185. Zaragoza, M. M.; Sankaran-Walters, S.; Canfield, D. R.; Hung, J. K.; Martinez, E.; Ouellette, A. J.; Dandekar, S. Persistence of gut mucosal innate immune defenses by enteric alpha-defensin expression in the simian immunodeficiency virus model of AIDS. *J. Immunol.* **2011**, *186*, 1589–1597.

186. Matsushita, I.; Hasegawa, K.; Nakata, K.; Yasuda, K.; Tokunaga, K.; Keicho, N. Genetic variants of human beta-defensin-1 and chronic obstructive pulmonary disease. *Biochem. Biophys. Res. Commun.* **2002**, *291*, 17–22.

187. Salvatore, F.; Scudiero, O.; Castaldo, G. Genotype-phenotype correlation in cystic fibrosis: the role of modifier genes. *Am. J. Med. Genet.* **2002**, *111*, 88–95.

188. Vankeerberghen, A.; Scudiero, O.; De Boeck, K.; Macek, M., Jr.; Pignatti, P. F.; Van Hul, N.; Nuytten, H.; Salvatore, F.; Castaldo, G.; Zemkova, D.; et al. Distribution of human beta-defensin polymorphisms in various control and cystic fibrosis populations. *Genomics* **2005**, *85*, 574–581.

189. Levy, H.; Raby, B. A.; Lake, S.; Tantisira, K. G.; Kwiatkowski, D.; Lazarus, R.; Silverman, E. K.; Richter, B.; Klimecki, W. T.; Vercelli, D.; et al. Association of defensin beta-1 gene polymorphisms with asthma. *J. Allergy Clin. Immunol.* **2005**, *115*, 252–258.

190. Dork, T.; Stuhrmann, M. Polymorphisms of the human beta-defensin-1 gene. *Mol. Cell Probes* **1998**, *12*, 171–173.

191. Braida, L.; Boniotto, M.; Pontillo, A.; Tovo, P. A.; Amoroso, A.; Crovella, S. A single-nucleotide polymorphism in the human beta-defensin 1 gene is associated with HIV-1 infection in Italian children. *Aids* **2004**, *18*, 1598–1600.

192. Milanese, M.; Segat, L.; Pontillo, A.; Arraes, L. C.; de Lima Filho, J. L.; Crovella, S. DEFB1 gene polymorphisms and increased risk of HIV-1 infection in Brazilian children. *Aids* **2006**, *20*, 1673–1675.

193. Baroncelli, S.; Ricci, E.; Andreotti, M.; Guidotti, G.; Germano, P.; Marazzi, M. C.; Vella, S.; Palombi, L.; De Rossi, A.; Giuliano, M. Single-nucleotide polymorphisms in human beta-defensin-1 gene in Mozambican HIV-1-infected women and correlation with virologic parameters. *Aids* **2008**, *22*, 1515–1517.

194. Milanese, M.; Segat, L.; Crovella, S. 2007. Transcriptional effect of DEFB1 gene 5' untranslated region polymorphisms. *Cancer Res.* 67:5997; author reply 5997.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

195. Ricci, E.; Malacrida, S.; Zanchetta, M.; Montagna, M.; Giaquinto, C.; Rossi, A. D. Role of beta-Defensin-1 Polymorphisms in Mother-to-Child Transmission of Human Immunodeficiency Virus Type 1. *J. Acquired Immune Defic. Syndr.* **2009**.
196. Zapata, W.; Rodriguez, B.; Weber, J.; Estrada, H.; Quinones-Mateu, M. E.; Zimermman, P. A.; Lederman, M. M.; Rugeles, M. T. Increased levels of human beta-defensins mRNA in sexually HIV-1 exposed but uninfected individuals. *Curr. HIV Res.* **2008**, *6*, 531–538.
197. Yang, C.; Boone, L.; Nguyen, T. X.; Rudolph, D.; Limpakarnjanarat, K.; Mastro, T. D.; Tappero, J.; Cole, A. M.; Lal, R. B. theta-Defensin pseudogenes in HIV-1-exposed, persistently seronegative female sex-workers from Thailand. *Infect. Genet. Evol.* **2005**, *5*, 11–15.
198. Tugizov, S. M.; Herrera, R.; Veluppillai, P.; Greenspan, D.; Soros, V.; Greene, W. C.; Levy, J. A.; Palefsky, J. M. HIV is inactivated after transepithelial migration via adult oral epithelial cells but not fetal epithelial cells. *Virology* **2011**, *409*, 211–222.
199. de Leeuw, E.; Lu, W. Human defensins: turning defense into offense? *Infect. Disord.: Drug Targets* **2007**, *7*, 67–70.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch007

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Chapter 8

"GENOPEP", a Topical Cream in the Treatment of Burn Wounds

Jesse M. Jaynes*,[1] and V. Siva Rami Reddy[2]

[1]Tuskegee University, Integrative Biosciences,
Tuskegee, AL 36811, United States
[2]Osmania Medical College, Plastic Surgery,
Hyderabad 500 095, India
***E-mail: jjaynes@mytu.tuskegee.edu**

The loss of the skin's protective barrier as the result of burns fosters the susceptibility to bacterial infection, invasion, and sepsis. Infection remains the leading cause of death among patients who are hospitalized for burns. Current standards of treating the burned tissue have severe limitations and inherent risks of complications. Based upon the principles discovered in naturally occurring peptides, recent designs of synthetically engineered antimicrobial peptides have demonstrated increased potency and efficacy/tolerability, enhanced specificity, and reduced toxicity in comparison to the extant burn treatment modalities. These peptides termed as designed antimicrobial peptides (dAMP), are resistant to such effects of high solute levels and demonstrate even greater antibacterial activity than traditional antibiotics. One such peptide, GENOPEP, has shown significant antimicrobial activity and accelerated wound healing and is the first such peptide to be used to treat burns in humans. The impact of this treatment could improve patient survival or quality of life and reduce costs to the patient, their family, hospital and society.

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Introduction

The loss of the skin's protective barrier as the result of burns fosters the susceptibility of the wound to bacterial infection, invasion, and sepsis. Infection remains the leading cause of death among patients who are hospitalized for burns. The risk of burn wound infection is directly related to the extent of the burn (first degree burn; second degree burn; third degree burn) and is related to impaired resistance due to disruption of the skin's mechanical integrity and generalized immune suppression (*1–3*).

Current standards of treating the burned tissue include applying topical antibiotics such as silver sulfadiazine, mafenide acetate, or silver nitrate to the burn wounds to help prevent massive bacterial invasion and sepsis, and use of oral or intravenous antibiotics. Unfortunately, each of these agents has its limitations and inherent risk of complications (*4–7*).

The use of silver sulfadiazine, for example, has been demonstrated to increase wound epithelialization but can impair wound contraction (*8*). Mafenide acetate has been demonstrated to enhance angiogenesis, epithelialization, and dermal thickening in some studies, while in others it has been linked to decreases in keratinocyte growth rates and is a known source of metabolic acidosis through its inhibition of carbonic anhydrase (*9*, *10*). Both of these agents have a limited spectrum of antibacterial activity.

Other topical agents used to decrease the wound bacterial load have included Dakin's (sodium hypochlorite) solution, betadine, acetic acid, and hydrogen peroxide. Dakin's solution exhibits deleterious effects to fibroblasts and endothelial cells and can impair neutrophil migration and wound neovascularization (*11*). Studies on Betadine have shown slower rates of re-epithelialization compared to other topical antimicrobial agents and impairment of microcirculation at higher levels of concentration (*12*). Acetic acid alternatively does not demonstrate effective control to keep bacterial levels at less than 10^5 colonies per gram of tissue and is cytotoxic at its traditionally used concentration of 0.25% (*13*) . Hydrogen peroxide can also be toxic to fibroblasts (*14*).

Oral or intravenous antibiotics are often used in conjunction with topical antimicrobials to decrease the bacterial burden on tissue. As more focus is centered on the problem of multi-drug resistant bacteria, choices for effective selection of antimicrobial agents can become limited. Methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin resistant Enterococcus faecium/faecalis (VRE) are two very resistant bacterial strains that are difficult to treat with current antibiotics (*15–17*).

Furthermore, these resistant bacteria have the potential of fostering cross-resistance through plasmid transfer (*18*). Transmission of multi-resistant organisms to other patients, particularly in contained burn units, not only increases morbidity, but also adds an enormous cost to the hospitals and society (*19*, *20*). One percent of all patient discharges from the hospital have ongoing Staphylococcus aureus infections (*21*). The hospital costs for people with Staphylococcus aureus infections were twice those of other patients (*22*). Clearly the need for effective antimicrobial agents is urgent as drug resistance continues to emerge.

It is clear that the topical agents are crucial in the ultimate eradication of the burn and infected wound pathogens since it is extremely difficult to administer the intravenous antibiotics to non-perfused tissue such as burned skin. The poorly vascularized, burned skin is, therefore, the portal of entry and the ongoing nidus of infection for burn victims. The ideal topical agent should be highly active against common and multi-resistant pathogens, such as methicillin resistant Staphylococcus aureus, vancomycin resistant Enterococcus faecium/faecalis, and extended spectrum β-lactamase producing Gram-negative organisms, while having a neutral or even beneficial effect on the wound healing process.

Antimicrobial peptides represent a relatively new discovery in the immune system pathway. These small peptides are inducible elements of the immune system that serve as nonspecific effector molecules to eradicate infection caused by bacteria, yeast, and viruses, protecting host epithelial surfaces such as the tracheal mucous membrane and genitourinary tract (*23–26*). In mammals, several of these compounds are known to be present in high concentrations in neutrophilic granules and phagocyte vacuoles. These peptides differ significantly in their structure between species but, in common, appear to create amphipathic helical or beta-pleated structures. The mechanism of action is different from currently utilized antibiotics and appears to be based on their ability to insert into membranes, from channels or “pores”, and destroy the cell by changing membrane conductance and altering intracellular function (*27*, *28*).

Based upon the principles discovered in the naturally occurring peptides, recent designs of synthetically engineered antimicrobial peptides have demonstrated increased potency and efficacy/tolerability, enhanced specificity, and reduced toxicity in comparison (*28–38*). These peptides termed as designed antimicrobial peptides (dAMP), are resistant to such effects of high solute levels and demonstrate even greater antibacterial activity (*39*). One such peptide, GENOPEP, has shown significant promise in in vitro studies against a large number of pathogens and is very solute resistant. GENOPEP is the trade name for a gel preparation containing the dAMP D2A21 that has been shown to improve survival and wound re-epithelization of full-thickness burns in rats compared to control treatments: vehicle, SSD and Sulfamylon (*40*, *41*).

This antimicrobial peptide shows sigificant promise in treating patients with chronic wounds or burn wound sepsis. The impact of this could improve patient survival or quality of life and reduce costs to the patient, their family, hospital and society (*40*, *41*).

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Methodology

In Vitro Antimicrobial Studies Using GENOPEP

The proprietary test compound ‘GENOPEP’ showed high antibacterial activity on test organisms Staphylococcus aureus MTCC 96 and Pseudomonas aeroginosa MTCC 741. The test compound showed 100 % killing of Staphylococcus aureus on exposure to 1 μM (4.3 μg/ml) and 5 μM (21.5 μg/ml) concentrations for 1 hr at pH 7.2, and at pH 8.4 an exposure of 4 hrs was required to get 100% killing. Whereas, 100% killing of Pseudomonas aeruginosa was observed on exposure to the test compound for 1 hr at pH 8.4 and an exposure of 4 hrs was required for 100% killing at pH 7.2.

The microbiological studies with GENOPEP in vivo using a rat burn wound model were conducted. The observations on the bacterial growth in eschar and sub-eschar muscles on post burn day one, two or three in peptide treated and control treated groups were made. A substantial decrease in the microbial population level was observed in animals treated with peptide (unpublished pre-clinical studies).

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Animal Studies Using GENOPEP

Sub-acute toxicity studies (conducted using well-established protocols) of GENOPEP in rats and rabbits demonstrated its safety when used topically. No abnormalities in physical, physiological, biochemical and histo-pathological parameters were observed by the topical application of the peptide. No mortalities in animals of any group were observed (unpublished pre-clinical studies).

There is evidence (dermal histopathology findings) to show that GENOPEP has stimulatory action on tissue growth (increased collagen content in granulation tissue and re-epithelialization) thus promoting improved wound healing (unpublished pre-clinical studies).

Phase-I Clinical Studies

The results of Phase-I clinical trial on healthy human patients revealed that GENOPEP cream administered topically twice a day was safe. GENOPEP was safe and adverse events were found to be minimal in the Phase-I Study. Treatment Groups were similar in efficacy/tolerability and safety parameters studied. With the conclusion of this study, GENOPEP cream was allowed to proceed to to Phase-II clinical trils as per Schedule Y (Amendment 2005) of Drugs and Cosmetics Rule 1940.

Phase II/III Study Design

The study was a double blind, randomized and placebo treatment controlled study in India. The study aimed to evaluate the efficacy of GENOPEP Cream in the treatment of burn wounds. Figure 1 describes the trial design in a schematic diagram.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Testing Procedures

Test Drug

GENOPEP 0.02% & GENOPEP 0.05%.

Placebo Treatment

GENOPEP Base

Dosing

Group 1: GENOPEP cream 0.02%, half a gram/cm2 applied every alternate day for 21 days or Healing of wound which ever was earlier.

Group 2: GENOPEP cream 0.05%, half a gram/cm2 applied every alternate day for 21 days or Healing of wound which ever was earlier.

Group 3: Placebo, half a gram/cm2 applied every alternate day for 21 days or Healing of wound whichever was earlier.

Method of Administration and Instructions for Use

Selected Patients instructed to report to the investigator every alternate day in the morning

Site of Application

Apply the prescribed treatment to the patient on wound area

Measurements (Area of Application)

Complete Wound Area

Procedure

After thorough cleaning of the site of application, the given formulation was applied uniformly in complete burn wound area. The site was covered with sterile pad and bandage. The patient was instructed to report any adverse event either to the investigator or the study personnel.

Duration of Treatment

21days (11 Visits) or Healing of the Burn Wound which ever was earlier.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

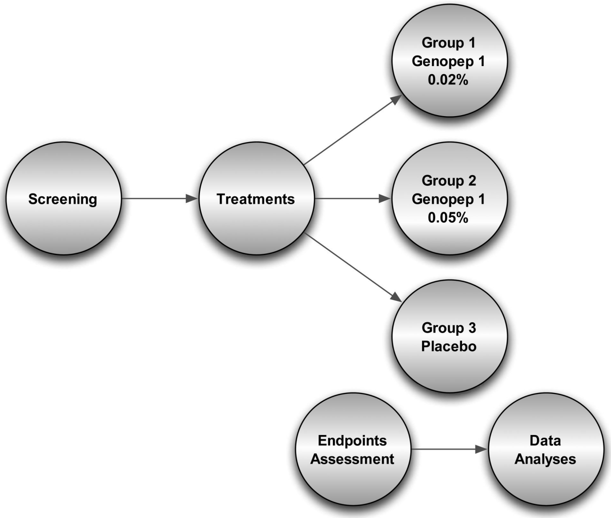


Figure 1. Trial design flow diagram.

Evaluation Criteria

Efficacy/Tolerability Variables

Primary End Point: The primary endpoint of the treatment was taken as complete closure / healing of the wound. At each visit form visit 2 the functional assessment of the wound was determined using the following scale below.

100 % wound closure: with complete epithelialization and no drainage or scab present

Less than 100% closure with drainage present.

The primary efficacy criteria are defined as the percentage of patients achieving a complete wound closure (functional assessment of score of 0) within the three-week treatment period. If a score of 0 is achieved for any patient then the medication will be stopped and recorded in the CRF as having reached the primary endpoint.

In addition to complete closure of the wound the endpoint of the treatment also considered the following:

Extent of non-viable tissue by clinical evaluation % of wound covered with non-viable tissue

- 76-100%
- 51-75%
- 26-50%
- 1-25%
- No Necrotic Tissue

Degree of granulation by visual Score % of wound filled with granulation tissue

- No Granulation
- Scanty Granulation
- Healthy Granulation

Besides the above parameters for an assessment for the primary efficacy, the secondary efficacy is assessed based on average wound evaluation score.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Wound Evaluation Score

Wound Evaluation Done on Four Parameters:

- Erythema (redness of the skin caused by dilatation and congestion of the capillaries, often a sign of inflammation or infection)
- Edema (excessive accumulation of serous fluid in tissue spaces)
- Purulence (the state or condition of containing or secreting pus)
- Necrotic Tissue (dead, devitalized tissue)

Each of these parameters is measured on a scale of 0-3 as follows: 0 = Absent; 1 = Mild; 2 = Moderate; 3 = Severe.

A Wound Evaluation Score (WES) of 0 is considered as a secondary efficacy criterion. The closer this score is to 0 the more significant the healing and revitalization of the wound.

Statistical Methods

The study aims to evaluate the safety and efficacy of treatment in three groups. The primary end point parameters of patients with epithelialization/healing of wound in different groups was assessed and analyzed by $\chi 2$ test to hypothesis testing between groups to measure the efficacy of the test groups and for the complete healing of patients. The Secondary efficacy variable being a categorical variable, the difference was analyzed by $\chi 2$ test. Safety analysis with $\chi 2$ test for categorical variables and GLM (ANOVA) for continuous variables were conducted. Statistical significance was considered when P value is < 0.05.

Assessment Schedule

After screening, the patients were allotted to Treatment Groups as per the randomization schedule. The assessment schedule for all three groups was the day of reporting burn wound i.e. on 0th day, 12th day and 20th day. The maximum number of visits was expected were 11 during the study period of 21 days. The assessment schedule, major study milestones and drug description are given in Figure 2 and Tables I & II, respectively.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Table I

Step	***Milestone***	***Dates***
1	Filing of Clinical Trial Protocol	August 2007
2	Clinical Trial Protocol Approval	August 2007
3	Investigators Meeting	October 2007
4	IRB/EC Approval	November 2007
5	Site Initiation	November 2007
6	Patient Screening and Recruitment	November 2007
7	Last Patient In	September 2008
8	Last Patient Out	September 2008
9	Trial Report	March 2010
10	Report Submission	May 2010

Table II

Item	***Description***
Study Drugs	GENOPEP Cream 0.02%, 0.05% and Base
Manufacturer	ISSAR Pharmaceuticals
Purity	97.8%
How Supplied	5 gm tubes
Precautions	Test at Room Temperature
Shelf Life	24 Months at Room Temperature
Route of Administration	Topical Cream
Dosing	Sufficient for Burn Wound
Contraindications	Nil
Drug Interactions	None
Use During Pregnancy	Can be Used
Drug Supplies & Labels	Yes as per Stipulated Guidelines
Drug Accountability	Yes
Intercurrent Illness	Yes

Assessment Schedule

The study was conducted at Osmania General Hospital, Hyderabad on 60 patients. Twenty patients each on 0.02% & 0.05% peptide containing cream and Placebo Treatment Groups formed the study samples.

Criteria for Inclusion or Exclusion

Inclusion Criteria:

- Adult male or female patients aged above 18 years of age.
- Patients with partial thickness burn wounds.
- Total surface area of the burn less than 20%
- Willing to give written informed consent.

Exclusion Criteria

- Patients with more than 20% of burns.
- Patients with full thickness burns
- Patients who need skin grafting.
- Patients with diabetes.
- Immune compromised patients.
- Patients with infectious diseases.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Disposition of Subjects

The efficacy data was analyzed for evaluable patients. Table III shows the number of subjects and the reasons for excluding the subjects from the data set for evaluable subjects. Thus, a total of 60 subjects were included and completed this study.

Table III. Disposition of Subjects

	0.02% GENOPEP	*0.05% GENOPEP*	*Placebo*
Number Treated	20	20	20
Non Compliance	0	0	0
Efficacy/Tolerability	20	20	20
Number Completed	20	20	20
Number Withdrawn	0	0	0
Absence to Treatment	4	3	4

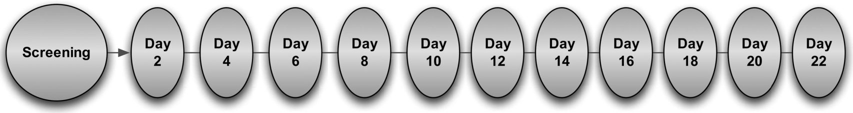


Figure 2. Days of assessment.

Demography

The data was analyzed at visit 1 (baseline) with respect to demographic characteristics. There was no significant statistical difference observed between Drug groups in the parameters such as Age, Weight and Height. The Age group ranged from 18 to 72 years, the majority belonged to age group 18-48 years, the weight ranged from 40 kg to 92 kg and height ranged from 142 cm to 176 cm (Table IV).

Table IV. Demography of Subjects

Group	*N*	*Variable*	*Mean*	*SD*	*Minimum*	*Maximum*
GENOPEP-0.02%	20	Age Weight Height	29.35 60.15 159.25	9.40 11.44 7.85	18.00 44.00 142.00	48.00 84.00 176.00
GENOPEP-0.05%	20	Age Weight Height	28.35 61.80 159.65	7.32 13.37 6.92	18.00 46.00 148.00	45.00 89.00 174.00
Placebo	20	Age Weight Height	31.90 64.15 160.60	11.41 12.51 6.49	18.00 48.00 146.00	55.00 92.00 175.00

Burn Characteristics

The characteristics of the burns at baseline (visit-1) are presented in Table V below. Nearly 90% had multiple burns. As per inclusion criteria only patients with ≤20% burn were selected into the study and were assigned at random to the treatment groups. The percent of the burns ranged from 3 to 20%. The average burn size was 17.25% in the GENOPEP 0.02% group, 15.55% in the GENOPEP 0.05 % group and 18.75% in the Placebo Group of the total body surface area for each patient. By Analysis of Variance (ANOVA) the group means were found to be statistically non-significant. Thus indicating the groups were similar in burn characteristics at visit-1.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Table V. Mean Percentage of Burns by Group

Group	*N*	*Mean*	*SD*	*Minimum*	*Maximum*
GENOPEP-0.02%	20	17.25%	4.70	3.00	20.00
GENOPEP-0.05%	20	15.55%	6.26	3.00	20.00
Placebo	20	18.75%	3.02	9.00	20.00

Results and Conclusions

Primary Efficacy/Tolerability Conclusions

Primary efficacy assessment was carried out on the patients with epithelialization/healing of the wound. Statistical significance was considered at P<0.05 assuming a null hypothesis that the efficacy parameter was significantly different among Treatment Groups. To determine the effective dose and regimen, the above analysis was performed between placebo, 0.02% Peptide and 0.05% Peptide Treatment Groups.

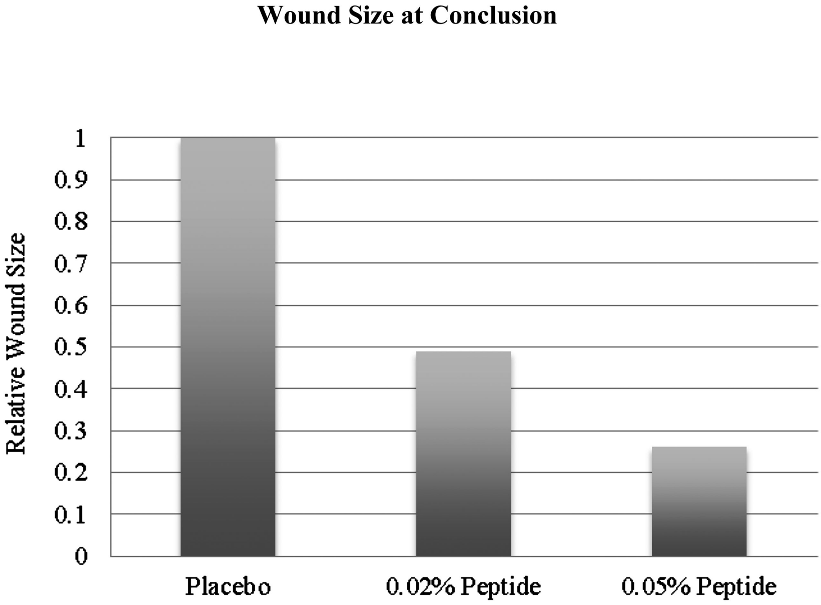


Figure 3. Both peptide treated groups achieved accelerated wound healing from that of the placebo with a greater level of significance than P<0.05 (0.02% peptide group P<0.011 and 0.05% peptide group P<0.0044).

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Table VI. Wound Evaluation Score

Group	***Ery-thema***		***Edema***		***Purulence***		***Necrosis***		***WES***		***Ave WES***	
Visit	First	Last	First	Last	First	Last	First	Last	First	Last	First	Last
0.02% Pep	44	1	21	0	0	3	60	7	125	11	6.25	0.55
0.05% Pep	50	0	17	0	0	2	60	5	128	7	6.40	0.35
Placebo	41	1	12	2	0	9	60	14	113	26	5.65	1.30

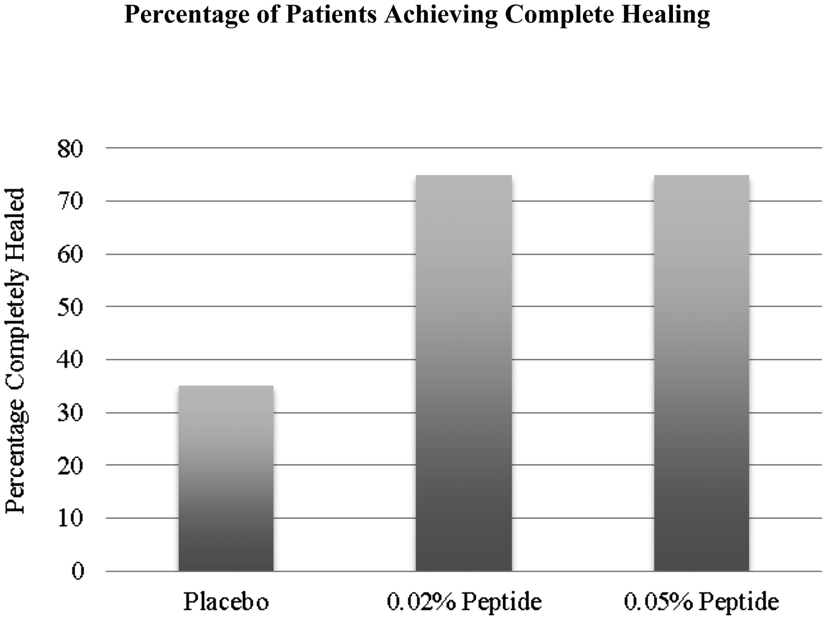


Figure 4. Both peptide treated groups achieved a greater number of patients that were completely healed than the placebo with a lower than P<0.05 level of significance (both 0.02% and 0.05% at P<0.011).

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

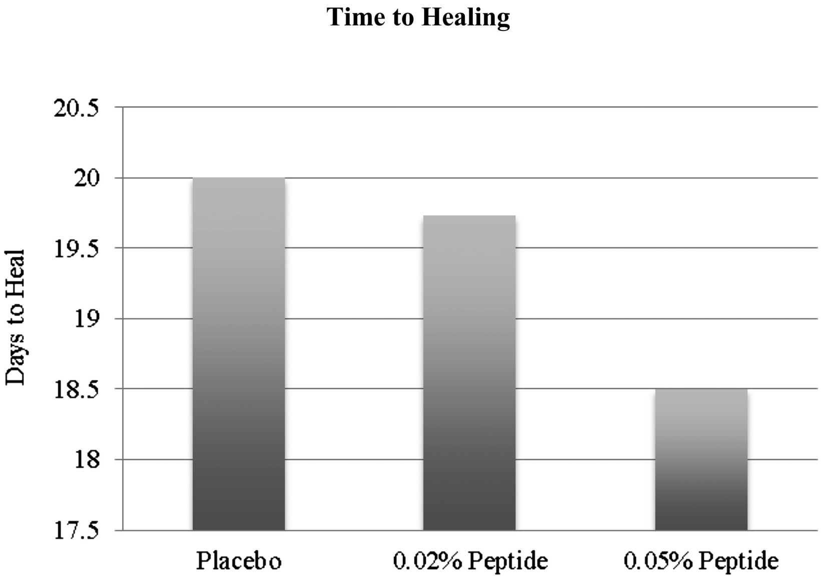


Figure 5. Both peptide treated groups achieved a lower average time of healing. A significant difference was seen between the 0.05% peptide group and Placebo treatment with a P<0.017.

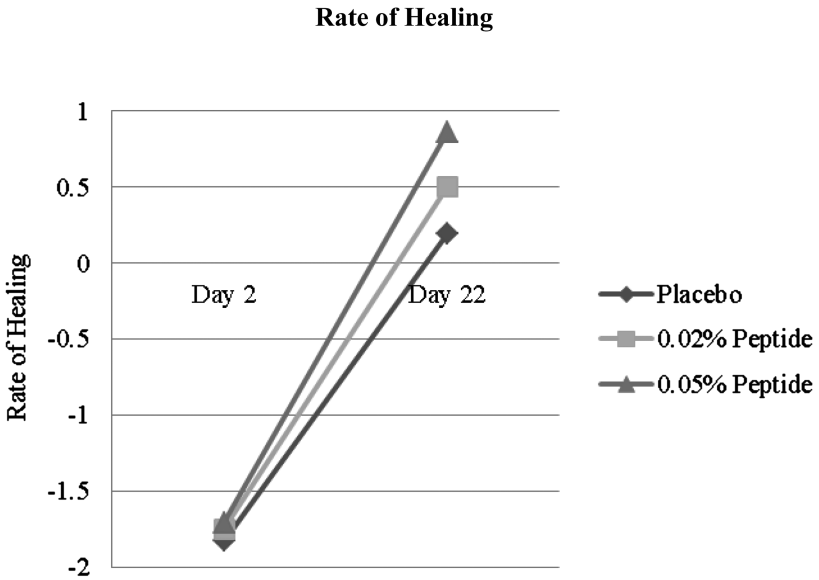


Figure 6. Both peptide treated groups achieved a lower average time of healing and an increase in the rate of wound closure than that of the Placebo. A significant difference was seen between the 0.05% peptide and Placebo Treatments P<0.05.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Secondary Efficacy/Tolerability Conclusions

Descriptive statistics were calculated for all the secondary efficacy variables and compared between groups at endpoint using Analysis of Variance for significance between groups at $P < 0.05$.

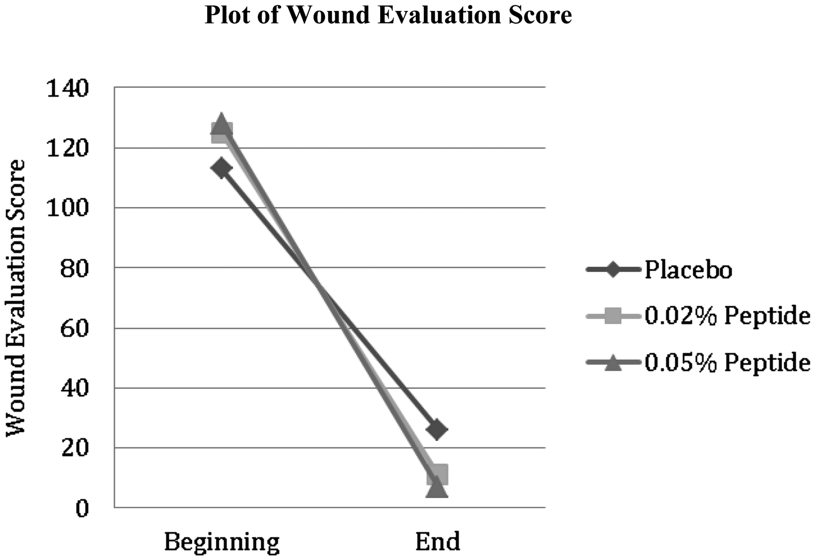


Figure 7. Wound Evaluation Scores of all groups were calculated and showed that there was statistical significance (for both groups P<0.011) between the peptide treated groups and the placebo group on the last day of evaluation (Table VI and The rate at which the WES changed over time) is shown.

The closer to 0 indicates more positive characteristics of wound healing with 0 being completely healed.

Figure 8 A and B demonstrate visually the difference between groups with A = Placebo, B = 0.02% Peptide and C = 0.05% Peptide.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

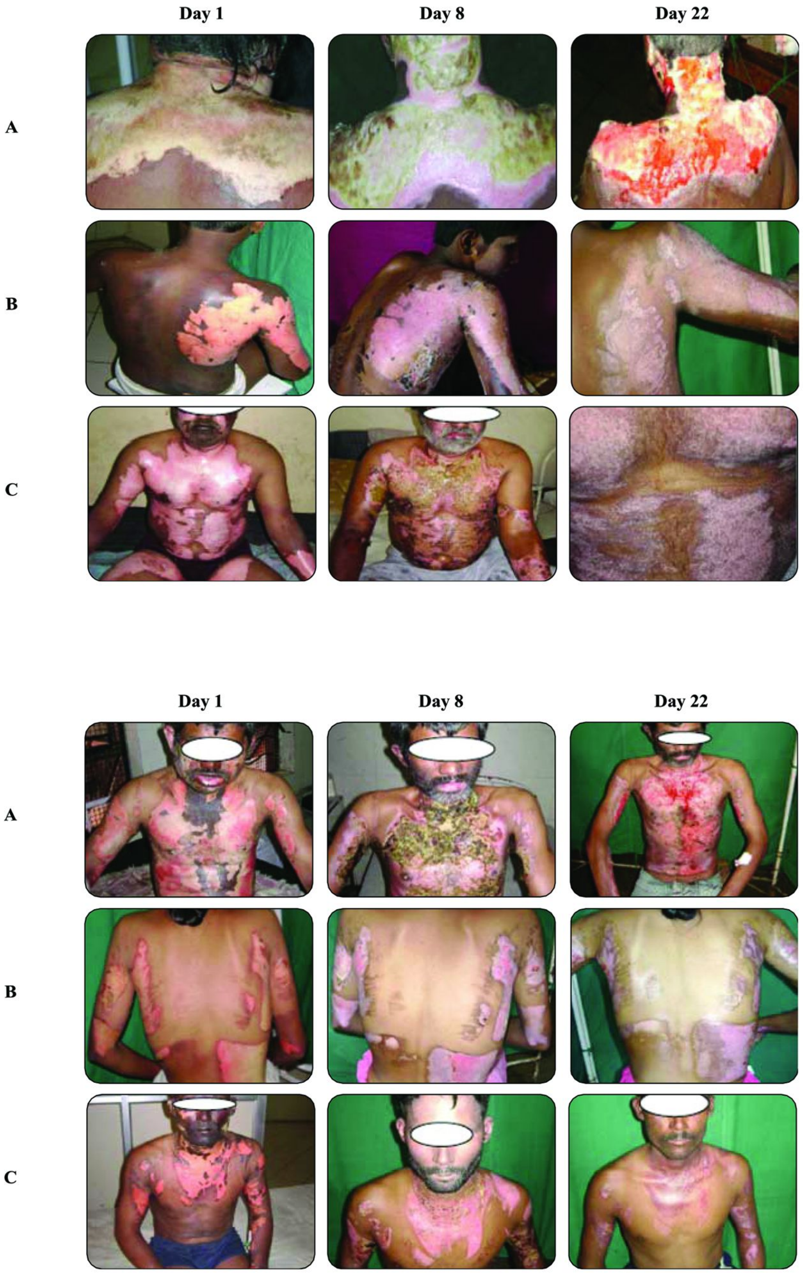


Figure 8. In A and B, the increased healing can be clearly seen in the 0.02% and 0.05% peptide groups while the Placebo Group is clearly lagging in healing rate. (see color insert)

Safety Conclusions

The lab investigations included standard hematology and biochemical parameters. These investigations were used to assess the safety of the product. These conclusions were reached at both hospitals

General Linear Model (GLM) analysis was done to test the hypothesis that the lab-investigations were similar between baseline and study termination and there was no statistically significant difference found. However, a significant change between baseline and study termination in the leukocytes was observed.

It was observed that except the one variable (Total Protein) others were statistically non significant among the Treatment Groups (GENOPEP 0.02 %, GENOPEP 0.05% & Placebo).

Hence the overall results indicate that the safety variables are similar between time points and between groups.

The Vital signs includes Blood Pleasure, Pulse Rate, Heart Rate, Respiratory Rate and Temperature. These vital signs were used to assess the safety of the product and two sets of vital sign measurements were taken, one at the time of the baseline (visti-1) and another at the time of the termination visit. The General Linear Model (GLM) analysis was done to test the hypothesis that the vital sign measures are similar between base line and study termination. In about 11 units in 0.02% GENOPEP and 8 units in GENOPEP 0.05% as well as Placebo Group's subjects of pulse and heart rate reduction were observed form the baseline to termination day, however, they were all within the normal range.

It was observed that none of the variables were statistically significant between the Treatment Groups. Hence the overall results indicate the safety variables were similar between time points and between groups.

The Pharmacokinetic evaluation showed that the drug was not absorbed into the system as it was not detected in the serum samples of patients.

Summary and Final Conclusions

These double blind studies were conducted on 60 patients who were above 18 years of age with less than or equal to 20% partial thickness burns. They were randomly divided into three study groups of 20 patients each. The primary end point taken was complete wound closure or complete healing of burns of study subjects and the secondary end point was added to assist in the complete wound healing of the patient.

Twenty five percent of the patients in the Placebo Group completely healed while, remarkably, 15 (75%) patients in the GENOPEP 0.02% Peptide Treatment Group and 15 (75%) patients in the GENOPEP 0.05% Peptide Treatment Group completely healed in the stipulated study time period.

It was also found that in the GENOPEP Peptide Treatment Groups, there was significantly decreased time to healing from that of the Placebo Groups.

In the GENOPEP Peptide Treatment Groups, the incidence rate of wound healing was better as the scar formation was significantly lower compared to that in the Placebo Group, indicating that treatment with GENOPEP enables better healing with less morbidity.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Treatment compliance was good and there were no side effects or adverse reactions or toxic effects noted in hematological or in biochemical tests with both study groups as compared with the Placebo Groups.

The pharmacokinetic samples, at 0 hr, 30 mins and study termination day, showed that there was no drug present in the sera found in patients at both study sites.

It is clear that the GENOPEP medication can be used as a long-term medication for burn patients without any side effects. It is concluded that the GENOPEP cream is safe and is highly effective in promoting burn wound healing (compared to vehicle control) for patients with partial thickness burns that are less than or equal to 20% without any side effects even if the drug is used as a longer term medication.

Therefore, it is worthwhile studying the efficacy and safety of the GENOPEP cream in both 0.02% & 0.05% forms in treating larger groups of burn patients with more than 20% partial thickness burns and comparing it to the standard treatment with SSD or Sulfamylon. These proposed studies are underway.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Acknowledgments

We would like to acknowledge the support of Issar Pharmaceuticals and Mr. Ram Isanaka in completion of the burn trial described in this manuscript.

References

1. Bennet, L. L.; Rosenblum, R. S.; Perlov, C. An in vivo comparison of topical agents on wound repair. *Plast. Reconstr. Surg.* **2001**, *108*, 675–687.
2. Boyce, S. T.; Supp, A. P.; Swope, V. P.; Warden, G. D. Topical Sulfamylon reduces engraftment of culture skin substitutes on athymic mice. *J. Burn Care Rehabil.* **1999**, *20*, 33–36.
3. Geronoemus, R. G.; Mertz, P. M.; Eaglstein, W. H. Wound healing : the effects of topical antimicrobial agents. *Arch. Dermatol.* **1979**, *115*, 1311–1314.
4. Kjolseth, D.; Barker, F. J.; Anderson, G. L. comparison of the effects of commonly used wound agents on epithelization and neovascularization. *J. Am. Coll. Surg.* **1994**, *179*, 305–312.
5. Leitch, I. O.; Kucukcelebi, A.; Robson, M. C. Inhibition of wound contraction by topical antimicrobials. *Aust. N. Z. J. Surg.* **1993**, *63*, 289–293.
6. Watcher, M. A.; Wheeland, R. G. The role of topical agents in the healing of full-thickness wounds. *J. Dermatol. Surg. Oncol.* **1989**, *15*, 1188–1195.
7. Lehrer, R. I.; Lichtenstein, A. K.; Ganz, T. Defensins ; Antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* **1993**, *11*, 105.
8. Gayle, W. E.; Mayhall, C. G.; Lamb, V. A.; Apollo, E.; Haynes, B. W. Resistant Enterobacter cloacae in a burn center; the infectiveness of silver sulfadiazine. *J. Trauma* **1978**, *18*, 317–323.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

9. Hoffmann, S. Silver sulfadiazine : An antibacterial agent for topical use in burns- a review of the literature. *Scand. J. Plast. Reconstr. Surg.* **1948**, *18*, 119–126.
10. Smoot, E. C., III; Kucan, J. O.; Graham, D. R.; Barenfanger, J. E. Susceptibility testing of topical antimicrobial against methicillin-resistant Staphylococcus aureus. *J. Burn Care Rehabil.* **1992**, *13*, 198–202.
11. Rodgers, G. L.; Mortensen, A. E.; Fisher, M. C.; Lon, S. S. In Vitro susceptibility testing of topical anti-microbial agents used in pediatric burn patients : Comparison of two methods. *J. Burn Care Rehabil.* **1997**, *18*, 406–410.
12. Kozol, R. A.; Gillies, C.; Elgebaly, S. A. Effects of sodium hypochlorite (Kakin's solution) on cells of the wound module. *Br. J. Surg.* **1958**, *72*, 780–782.
13. Lineaweaver, W.; Howard, R.; Soucy, D. Topical antimicrobial toxicity. *Arch. Surg.* **1985**, *120*, 267–270.
14. Lineaweaver, W.; McMorris, S.; Howard, R.; Soucy, D. Cellular and bacterial toxicities of topical anti-microbial. *Plast. Reconstr. Surg.* **1985**, *75*, 394–396.
15. Rubin, R. J.; Harrington, C. A.; Poon, A. The economic impact of Staphylococcus aureus infection in New York City hospitals. *Emerg. Infect. Dis.* **1999**, *5* (1).
16. Berhe, M.; Edmond, M. B.; Bearman, G. Measurement and feedback of infection control process measures in the intensive care unit: Impact on compliance. *Am. J. Infect. Control* **2006**, *34*, 537–539.
17. Bhalla, A.; Pultz, N. J.; Gries, D. M. Acquisition of nosocomial pathogens on hands after contact with environmental surfaces near hospitalized patients. *Infect. Control Hosp. Epidemiol.* **2004**, *25*, 164–167.
18. Dunagan, W. C.; Murphy, D. M.; Hollenbeak, C. S.; Miller, SB Making the business case for infection control: pitfalls and opportunities. *Am J. Infect Control* **2002**, *30*, 86–92.
19. Kuehnert, M. J.; Hill, H. A.; Kupronis, BA Methicillin-resistant Staphylococcus aureus-related hospitalizations, United States. *Emerg. Infect. Dis.* **2005**, *11*, 868–872.
20. Murphy, D., Whiting, J. and Hollenbeak, C. S. *Dispelling the Myths: The True Cost of Healthcare-Associated Infections*; APIC Briefing; February 2007.
21. Lederer, S. R.; Riedelsdorf, G.; Schiffl, H. Nasal carriage of methicillin resistant Staphylococcus aureus: The prevalence, patients at risk and the effect of elimination on outcomes among outclinic haemodialysis patients. *Eur. J. Med. Res.* **2007**, *12*, 284–288.
22. Klevens, R. M.; Edwards, J. R.; Andrus, M. L.; Peterson, K. D.; Dudeck, M. A.; Horan, T. C. Dialysis surveillance report: National Healthcare Safety Network (NHSN)-data summary for 2006. *Semin Dial.* **2008**, *21*, 24–28.
23. Boman, H. G. Antibacterial peptides: key components needed in immunity. *Cell* **1991**, *65*, 205–207.
24. Goldman, M. J.; Anderson, G. M.; Stolzenberg, E. D.; Kari, U. P.; Zasloff, M.; Wilson, J. M. Human beta-defensin-1 is a salt-sensitive

antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **1997**, *88*, 553–560.

25. Lawyer, C.; Pai, S.; Watabe, M.; Bakir, H.; Eagleton, L.; Watabe, K. Effects of synthetic form of tracheal antimicrobial peptide on respiratory pathogens. *J. Antimicrob. Chemother.* **1996**, *37*, 599–604.
26. Lehrer, R. I.; Ganz, T.; Selsted, M. E. Defensins: endogenous antibiotic peptides of animal cells. *Cell* **1991**, *64*, 229–230.
27. Boman, H. G. Antibacterial peptides: basic facts and emerging concepts. *J. Intern. Med.* **2003**, *254*, 197–215.
28. Hill, C. P.; Yee, J.; Selsted, M. E.; Eisenberg, D. Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. *Science* **1991**, *251*, 1481–1485.
29. Gunshefski, L.; Macsai, M.; Granus, V.; Jaynes, J. Synthetic Cecropins (Shiva-11) Effective Against Gentamicin Resistant Organisms. *Invest. Ophthal. Vis. Sci.* **1993**, *34* (4), 850.
30. Jaynes, J. M.; Burton, C. A.; Barr, S. B.; Jeffers, G. W.; Julian, G. R.; White, K. L.; Enright, F. M.; Klei, T. R.; Laine, R. A. In Vitro Effect of Novel Lytic Peptides on Plasmodium falciparum and Trypanosoma cruzi. *FASEB* **1988**, *2* (13), 2878–2883.
31. Arrowood, M. J.; Jaynes, J. M.; Healy, M. C. In Vitro Activities of Lytic Peptides Against the Sporozoites of Cryptosporidium parvum. *Antimicrob. Agents Chemo.* **1991**, *35* (2), 224–227.
32. Barr, S. C.; Rose, D.; Jaynes, J. M. Activity of lytic peptides against intracellular Trypanosoma cruzi amastigotes in vitro and parasitemias in mice. *J. Parasit.* **1995**, *81* (6), 974–978.
33. Ballweber, L. M.; Jaynes, J. M.; Stamm, W. E.; Lampe, M. F. In Vitro Microbicidal Activity of Cecropin Peptides D2A21 and D4E1 and Gel Formulations containing 0.1-2.0% D2A21 against Chlamydia trachomatis. *Antimicrob. Agents Chemo.* **2002**, *46*, 34–41.
34. Ma, J.; Kennedy-Stoskopf, S.; Jaynes, J.; Tompkins, W. Inhibitory Activity of Synthetic Peptide Antibiotics on Feline Immunodeficiency Virus In Vitro. *J. Virol.* **2002**, *76*, 9952–9961.
35. Jaynes, J. M.; Jeffers, G. W.; Julian, G. R.; White, K. L.; Enright, F. M. In Vitro Cytocidal Effect of Lytic Peptides on Several Transformed Mammalian Cell Lines. *Pep. Res.* **1989**, *2* (2), 157–160.
36. Robertson, C.; Roberson, K.; Pinero, A.; Jaynes, J.; Paulson, D. Peptidyl membrane-interactive molecules are cytotoxic to prostate cancer cells in vitro. *World J. Urol.* **1998**, *16*, 405–09.
37. Arlotti, J. A.; Cimino, T. S.; Nguyen, T. S.; Dhir, R.; Thomas, A.; Jaynes, J. M.; Caldwell, A. L.; Getzenberg, R. H. Efficacy of a Synthetic Lytic Peptide in the Treatment of Prostate Cancer. *Urol. Oncol.* **2001**, *6*, 97–102.
38. Yates, C.; Sharp, S.; Jones, J.; Topps, D.; Coleman, M.; Aneja, R.; Jaynes, J.; Turner, T. LHRH-conjugated lytic peptides directly target prostate cancer cells. *Biochem. Pharm.* **2011**, *81*, 104–111.
39. Schwab, U.; Gilligan, P.; Jaynes, J.; Henke, D. In Vitro Acitivit,ies of Designed Antimicrobial Peptides against Multidrug-Resistant Cystic Fibrosis Pathogens. *Antimicro. Agents Chemo.* **1999**, 1435–1440.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

40. Chalekson, C. P.; Neumeister, M. W.; Jaynes, J. M. Treatment of Infected Wounds with Antimicrobial Peptide, D2A21 (Demegel). *Plast. Reconstr. Surg.* **2002**, *109*, 1338–1343.
41. Chalekson, C. P.; Neumeister, M. W.; Jaynes, J. M. Improvement in Burn wound Infection and survival with Antimicrobial Peptide D2A21. *J. Trauma: Inj., Infect., Crit. Care* **2003**, *54* (4), 770–774.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch008

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

Chapter 9

Developing Influenza Antigen Microarrays for Seroprofiling

Yu M. Foong[1] and Mahesh Uttamchandani*,[1,2,3]

**[1]Department of Biological Sciences, Faculty of Science, 14 Science Drive 4,
National University of Singapore, Singapore 117543
[2]DSO National Laboratories, 27 Medical Drive, Singapore 117510
[3]Department of Chemistry, Faculty of Science, 3 Science Drive 3,
National University of Singapore, Singapore 117543
*Tel: (65) 6485 7214. Fax: (65) 6775 9011. E-mail: mahesh@dso.org.sg;
mahesh@nus.edu.sg**

Peptide and protein microarrays remain under-exploited tools in dissecting the immunogenic profiles of infections. Though antibody arrays have been conveniently developed for multiplexed detection of pathogenic contaminants in biological and environmental samples, it has perhaps been a greater challenge to produce antigen arrays, containing immunogenic peptide epitopes, for the detection of host exposure to infection. Herein we describe the development of such an antigen microarray platform against influenza, for potential applications in diagnostics, epitope mapping and potentially vaccine development. We have thus far successfully expressed a collection of 8 haemagglutinins (HAs) and 2 H1N1 targets for immobilization onto microarrays. This has been successful in yielding microarray binding profiles with anti-influenza antibodies spiked in human serum. We aim to expand on our antigen panel and build a wider influenza proteome array to aid in the serodiagnostics of influenza infections.

Keywords: Protein and peptide microarrays; Influenza; Antigen; Seroprofiling; Vaccine development; Epitope mapping

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

1. Introduction

The swine-origin Influenza A (H1N1) which emerged in Mexico and United States in April 2009 sparked a massive global pandemic. This variant strain contained gene segments which were traced to H1N1 (1918), 'avian-like' swine H1N1(1979) and human H3N2(1998) (*1*, *2*). Due to its rapid human-to-human transmission, WHO raised its pandemic alert to the highest level: 6, within two months of its emergence. Influenza's segmented genome and its wide host range favours evolution through antigenic drift and shift, producing novel strains of unknown virulence. In view of this rapid evolution and high transmissibility, fast, straightforward and sensitive detection platforms are required for precise diagnosis in times of a pandemic. Herein, we present the construction of an influenza protein (antigen) microarray to profile for exposure to the virus, which complements classical DNA diagnostic technologies.

Microarrays comprise high density assemblies of molecules spatially addressed across a planar surface (*3*). The x and y position of the spots on the microarrays denotes their identity, and the molecules of interest may either be fabricated *in situ* on the microarray, or fabricated elsewhere and then spotted robotically. The technology today allows the various different types of microarrays (DNA, protein, small molecule and so forth) to be conveniently mass produced, while, in general, requiring very low quantities of samples and reagents. One standard 3 inch by 1 inch glass slide can hold a large collection of different samples/ analytes, ranging from the tens of thousands to millions, depending on the fabrication method and intended application. As a result, microarrays provide a highly efficient test-bed for high-throughput screening.

Protein microarrays have over the years witnessed great success in a diverse range of applications. The first protein microarrays were developed by MacBeath *et al.* in 2000, which were applied in the study of protein-protein and protein-ligand interactions (*4*). Since then, several groups exploited this platform, with a variety of ligands and antibodies to target bacterial and viral pathogens in clinical and environmental samples (*5*, *6*) as well as in aiding the diagnosis of diseases (*7*, *8*).

Robinson *et al.* developed a diagnostic and profiling autoantigen array with 196 distinct autoantigens including DNA binding proteins and histones to detect and profile autoantibodies in serum samples from a variety of autoimmune diseases, including systemic lupus erythematosus (SLE), polymyositis (PM) and primary biliary cirrhosis (PBC) patients (*9*). In another application, Zhu *et al.* demonstrated the use of antigen microarrays for pathogen detection through the construction of a coronavirus proteome array to detect the immunological respone of patients infected with the Severe Acute Respiratory Syndrome (SARS) coronavirus using anti-human IgG (*10*). In other reports, a four- to eight-fold enhanced sensitivity was also demonstrated with microarrays, over conventional ELISA technologies (*9*), and this can be attributed to the reduced size features on microarrays (*11*). These properties, coupled with ability to multiplex targets, make microarrays a highly promising diagnostic platform (*12*). Epitope mapping has also been performed using peptide microarrays to identify the immunogenic epitopes (*13*–*15*). Using the most preferred binding epitopes of antigens, Parker

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

et al. developed an epitope-driven, imunotherapeutic vaccination strategy against *Mycobacterium tuberculosis* (*16*).

The concept of a diagnostic antigen microarray can potentially be extended to multiple viruses and bacterial pathogens. We herein describe the construction of an Influenza A antigen microarray for seroprofiling (Figure 1). This involves first cloning of the target proteins, expression and purification, ideally in a well-folded soluble form representative of its native structure, and then immobilizing it in a manner that preserves epitope accessibility on the microarrays. Thereafter, purified antibodies and/or infected serum is applied, followed by detection through labelled primary/secondary antibodies. In addition to applications in diagnostics, such protein microarrays can be applied to identify immunogenic protein(s) as well as epitopes within the viral proteome, which could trigger antibody production for protective immunity (*17*).

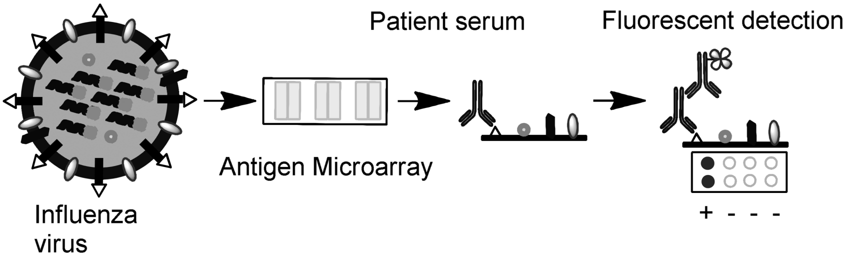


Figure 1. Working principles of an antigen microarray.

2. Materials and Methods

2.1. Protein Expression and Purification

Clones of A/New York/1682/2009(H1N1) were obtained from the Pathogen Functional Genomics Resource Center at the J Craig Venter Institute. These clones were eventually transformed into BL-21AI bacterial expression hosts. Postive transformants were grown in LB broth supplemented with 100µg/ml ampicillin to an OD_{600} of 0.6–0.8, upon which the expression was induced using 0.4% arabinose, and the cells were allowed to grow a further 4 h. The cell pellet from 50 ml of culture was lysed with lysozyme and pulse sonication (performed on ice), releasing the intracellular proteins into the lysis buffer (comprising 0.5% Triton-X, 10% glycerol, 0.1mM imidazole in phosphate buffered saline, PBS).

The haemagglutinin (HA) proteins H1-H8 were expressed extracellularly in Human Embryonic Kidney (HEK) 293T grown in DMEM media supplemented with 10% FBS (Gibco). 10µg of plasmid DNA was transfected for every T-75 flask at near 80% confluence using lipofectamine-2000 in the ratio of 0.4µg plasmid: 1µl lipofectamine. The resulting His-tagged proteins of interest were secreted into the media, and collected after periodic 48h harvests .

Proteins were purified by affinity-captured on Nickel-nitrilotriacetic acid resin (Qiagen), using protocols as recommended by the manufacturers, and eluted with 250mM imidazole. Typical proteins yields are described in Table 1, and purities, as approximated from the coomassie gels, were 85% and above (Figure 2).

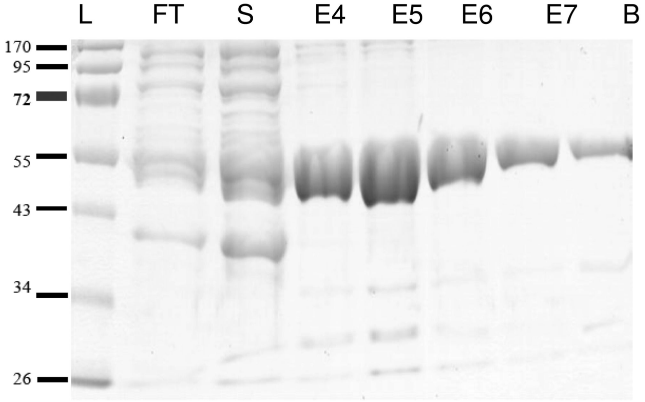


Figure 2. Purification of NS2 (MW = 46 kD) from 50ml of induced culture. L: ladder; FT: flow-through; S: supernatant; E: eluate; B: beads after 12 elutions.

2.2. Antigen Microarray Fabrication

Purified influenza proteins were diluted in 0.1M $NaHCO_3$, and were spotted at 0.1mg/ml concentrations on N-hydroxysuccinimide (NHS) -modified glass slides [for slide fabrication protocols, see (*18*)]. Grids were printed using SMP8B pins (Telechem International), with an inter-spot displacement of 1mm, on an GeneMachine spotter (Omnigrid, USA). Casein, lysozyme, bovine serum albumin (BSA) and avidin were co-spotted as negative controls on the microarrays. Upon spotting, the slides were incubated for 8h, quenched with 0.5M glycine in PBS (pH 7.4) for 20 min, rinsed with water, dried and stored at 4°C until required for use.

2.3. Antibody Labelling

Antibodies used were as follow: mouse monoclonal anti-His (Qiagen, Cat #: 34660), goat polyclonal anti-NS2 (Santa Cruz Biotechnology, Cat #: SC-17598) and rabbit polyclonal anti-HA (Pierce,Cat #: PA1-23094). A 45 µl labeling mix was set-up using 6 µg of antibody in 0.1M $NaHCO_3$ (pH 9.0), reacted with 1 µl of Cy3 or Cy5 monoreactive dye (GE Healthcare). After an hour on ice, the unreacted esters were quenched with 5µl of 1.0M Tris-Cl (pH 8.0). All 50µl was applied to Sephadex G-25 spin columns (GE Healthcare). The flow-through obtained was the labeled-antibody, as confirmed by fluorescent gel imaging (data not shown).

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

2.4. Antibody Application

Fluorescently labelled antibodies were diluted to the stated concentrations with PBS containing 1% BSA, to a final volume of 100µl and were applied to the slide under coverslip. The slides were then washed with water or PBST (PBS containing 0.1% tween), as necessary, to optimize signal to background, and scanned using an Axon 4000B microarray scanner at laser settings of 600 PMT and 100% power. Array data was extracted using the Genetix Pro 4.0 software.

Cy3-labeled anti-HA was also spiked into serum to a final concentration of 0.2 mg/ml and applied similarly. To improve signal to background ratios, the slide was scanned at 700 PMT and 100% laser power.

3. Results and Discussion

We tested the concept of antigen microarray with NS1, NS2 and H1 to H8 and commercially available antibodies. Table 1 summarises the vectors, tag(s) appended, expression host and yields of these proteins from his-tag purification (from a typical harvest of 50 ml of bacterial cell culture or from 5 T-75 flasks).

Table 1. Summary of successfully expressed proteins

Proteins[1]	*Vectors*	*Tags appended*	*Expression host*	*Total yields (µg)*
NS1	pLIC-cHalo	N-terminal his-tag; C-terminal halo-tag	*E. coli* BL21-AI	680
NS2				980
H1	pXJ	N-terminal his-tag	HEK 293T	200
H2	pXJ			275
H3	pTT5			260
H4	pTT5			245
H5	pXJ			255
H6	pXJ			280
H7	pTT5			335
H8	pTT5			305

[1] Molecular weights of proteins were as follows: NS1 – 58kD, NS2 – 46kD, H1-H8, 75kD.

His-tag NS1, NS2 and H1 to H8 were spotted on the NHS slides and probed with Cy5-labeled anti-His. These proteins were shown to be successfully immobilized (Figure 3). Cy3-labeled anti-NS2 binds to both NS1 and NS2 (Figure 4). It did not bind to H5 or any of the other control proteins. A similar selectivity pattern was also seen on the western blot (data not shown). This clearly indicates that antibodies can react to multiple proteins, which contain similar epitopes.

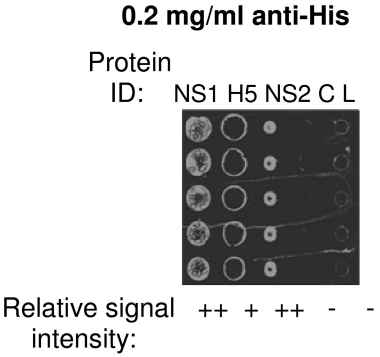

0.2 mg/ml anti-His
Protein ID: NS1 H5 NS2 C L
Relative signal intensity: ++ + ++ - -

Figure 3. Microarray detection using anti-His; target proteins were spotted on the arrays at a concentration of 75µg/ml, and antibodies were applied at 0.2 mg/ml in PBS containing 1% BSA. C – casein, L – lysozyme acted as negative controls.

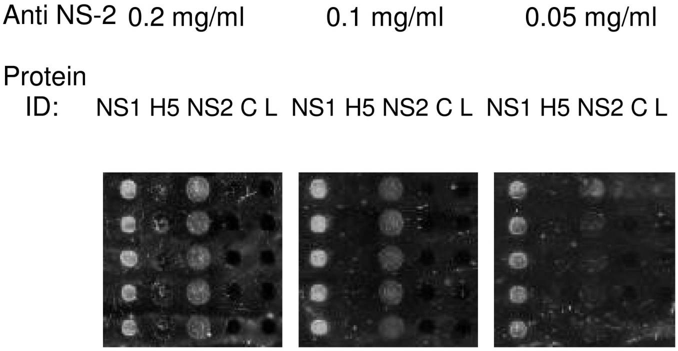

Anti NS-2 0.2 mg/ml
0.1 mg/ml
0.05 mg/ml
Protein ID: NS1 H5 NS2 C L NS1 H5 NS2 C L NS1 H5 NS2 C L

Figure 4. Microarray detection using anti-NS2 in a concentration dependent manner; target proteins were spotted on the arrays, in the order of NS1, H5, NS2, C (casein) and L (lysozyme), at a concentration of 75µg/ml, and antibodies were applied at 0.2 mg/ml, 0.1 mg/ml and 0.05 mg/ml in PBS containing 1% BSA respectively.

Encouraged by this results, we printed haemagglutinin proteins, subtypes H1-H8 on another array, albeit at lower concentrations. Out of the eight HAs spotted, Cy3-labeled anti-HA raised against avian strains bound to both H1 and H5 (Figure 5) but none of the other haemagglutinin subtypes. This cross-reactivity

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

with the commercial antibody was also confirmed by western blot, demonstrating that microarrays could be used as a platform to conveniently establish antibody selectivity. Weak or negligible background signals were obtained against the other haemagglutinin subtypes.

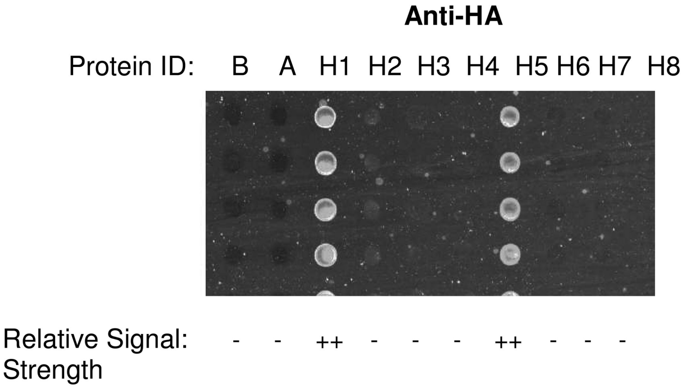

Anti-HA
Protein ID: B A H1 H2 H3 H4 H5 H6 H7 H8
Relative Signal: Strength - - ++ - - - ++ - - -

Figure 5. Microarray detection using anti-HA; target proteins are spotted at a concentration of 18.75 µg/ml, and antibodies are applied to serum at 0.2 mg/ml. B – BSA, A – avidin acted as negative controls.

Even with a low protein application concentration of 18.75µg/ml, we were able to immobilize sufficient antigens for detection by fluorescently labeled antibodies. Due to the cross-reactive nature of antibodies, antigen microarrays may however be less specific compared to classical detection techniques like real-time polymerase chain reaction (RT-PCR) which is commonly used for novel strain detection. Nevertheless, such microarrays may be able to differentiate early from late stage infection through variations in immunoglobulin-M (IgM) and IgG responses during the course of infection. However, detection of IgM within hours of infection may not be possible, due to low levels of antibodies, unlike RT-PCR, which at the early stages of infections may be able to pick up high viral loads. In attempting to diagnose individuals who may have different exposure histories, a combination of both DNA and protein based-detection platforms could improve overall diagnostic accuracy.

As part of the future work to this project, we are working to successfully express the entire viral proteome, in order to be able to establish a more comprehensive array of influenza antigens. We have recently expanded our microarray with the collection of H9 to H16 proteins, that will contribute to the diversity of proteins available on the microarray. Work is underway to improve the sensitivity of the platform, by concentrating and applying higher concentrations of the antigens, so as to detect antibodies present at physiological concentrations, to profile patient sera from the recent H1N1 pandemic.

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

4. Conclusion

Protein (antigen) microarray detects for antibodies directed against the virus in the serum; this was illustrated with the construction of influenza antigen microarray with NS1, NS2, H1-H8 proteins. Large-scale preparation and storage of such arrays can provide added utility in times of pandemic, in an attempt to attribute and identify sources of infections by being able to study samples from multiple species. Although certain antigens on the microarrays may exhibit cross-reactivity with sera, it can provide a useful tool for the quick and easy detection of exposed and infected individuals. Furthermore, this concept of antigen microarrays can be extended to the diagnosis of many pathogens, enabling high throughput, parallel and multiplex screening of infections. Being able to map immunogenic epitopes can also be extended from such a platform, allowing us to consider targets for potential vaccines.

Acknowledgments

The authors thank the Pathogen Functional Genomic Resource Centre (PFGRC) for the H1N1 clones, Dr. Brendon John Hanson for the HA clones and Angeline Lim for helpful suggestions on the manuscript.

References

1. Smith, G. J.; Vijaykrishna, D.; Bahl, J.; Lycett, S. J.; Worobey, M.; Pybus, O. G.; Ma, S. K.; Cheung, C. L.; Raghwani, J.; Bhatt, S.; Peiris, J. S.; Guan, Y.; Rambaut, A. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* **2009**, *459* (7250), 1122–5.
2. Garten, R. J.; Davis, C. T.; Russell, C. A.; Shu, B.; Lindstrom, S.; Balish, A.; Sessions, W. M.; Xu, X.; Skepner, E.; Deyde, V.; Okomo-Adhiambo, M.; Gubareva, L.; Barnes, J.; Smith, C. B.; Emery, S. L.; Hillman, M. J.; Rivailler, P.; Smagala, J.; de Graaf, M.; Burke, D. F.; Fouchier, R. A.; Pappas, C.; Alpuche-Aranda, C. M.; Lopez-Gatell, H.; Olivera, H.; Lopez, I.; Myers, C. A.; Faix, D.; Blair, P. J.; Yu, C.; Keene, K. M.; Dotson, P. D., Jr.; Boxrud, D.; Sambol, A. R.; Abid, S. H.; St George, K.; Bannerman, T.; Moore, A. L.; Stringer, D. J.; Blevins, P.; Demmler-Harrison, G. J.; Ginsberg, M.; Kriner, P.; Waterman, S.; Smole, S.; Guevara, H. F.; Belongia, E. A.; Clark, P. A.; Beatrice, S. T.; Donis, R.; Katz, J.; Finelli, L.; Bridges, C. B.; Shaw, M.; Jernigan, D. B.; Uyeki, T. M.; Smith, D. J.; Klimov, A. I.; Cox, N. J. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* **2009**, *325* (5937), 197–201.
3. Uttamchandani, M.; Wang, J.; Yao, S. Q. Protein and small molecule microarrays: powerful tools for high-throughput proteomics. *Mol. Biosyst.* **2006**, *2* (1), 58–68.
4. MacBeath, G.; Schreiber, S. L. Printing proteins as microarrays for high-throughput function determination. *Science* **2000**, *289* (5485), 1760–3.

5. Uttamchandani, M.; Neo, J. L.; Ong, B. N.; Moochhala, S. Applications of microarrays in pathogen detection and biodefence. *Trends Biotechnol.* **2009**, *27* (1), 53–61.
6. Duburcq, X.; Olivier, C.; Malingue, F.; Desmet, R.; Bouzidi, A.; Zhou, F.; Auriault, C.; Gras-Masse, H.; Melnyk, O. Peptide-protein microarrays for the simultaneous detection of pathogen infections. *Bioconjugate Chem.* **2004**, *15* (2), 307–16.
7. Hartmann, M.; Roeraade, J.; Stoll, D.; Templin, M. F.; Joos, T. O. Protein microarrays for diagnostic assays. *Anal. Bioanal. Chem.* **2009**, *393* (5), 1407–16.
8. Neuman de Vegvar, H. E.; Robinson, W. H. Microarray profiling of antiviral antibodies for the development of diagnostics, vaccines, and therapeutics. *Clin. Immunol.* **2004**, *111* (2), 196–201.
9. Robinson, W. H.; DiGennaro, C.; Hueber, W.; Haab, B. B.; Kamachi, M.; Dean, E. J.; Fournel, S.; Fong, D.; Genovese, M. C.; de Vegvar, H. E.; Skriner, K.; Hirschberg, D. L.; Morris, R. I.; Muller, S.; Pruijn, G. J.; van Venrooij, W. J.; Smolen, J. S.; Brown, P. O.; Steinman, L.; Utz, P. J. Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat. Med.* **2002**, *8* (3), 295–301.
10. Zhu, H.; Hu, S.; Jona, G.; Zhu, X.; Kreiswirth, N.; Willey, B. M.; Mazzulli, T.; Liu, G.; Song, Q.; Chen, P.; Cameron, M.; Tyler, A.; Wang, J.; Wen, J.; Chen, W.; Compton, S.; Snyder, M. Severe acute respiratory syndrome diagnostics using a coronavirus protein microarray. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (11), 4011–6.
11. Templin, M. F.; Stoll, D.; Schrenk, M.; Traub, P. C.; Vohringer, C. F.; Joos, T. O. Protein microarray technology. *Trends Biotechnol.* **2002**, *20* (4), 160–6.
12. Mochon, A. B.; Jin, Y.; Kayala, M. A.; Wingard, J. R.; Clancy, C. J.; Nguyen, M. H.; Felgner, P.; Baldi, P.; Liu, H. Serological profiling of a Candida albicans protein microarray reveals permanent host-pathogen interplay and stage-specific responses during candidemia. *PLoS Pathog.* **2010**, *6* (3), e1000827.
13. Reineke, U.; Ivascu, C.; Schlief, M.; Landgraf, C.; Gericke, S.; Zahn, G.; Herzel, H.; Volkmer-Engert, R.; Schneider-Mergener, J. Identification of distinct antibody epitopes and mimotopes from a peptide array of 5520 randomly generated sequences. *J. Immunol. Methods* **2002**, *267* (1), 37–51.
14. Halperin, R. F.; Stafford, P.; Johnston, S. A. Exploring antibody recognition of sequence space through random-sequence peptide microarrays. *Mol. Cell Proteomics* **2010**, *10* (3), M110 000786.
15. Cretich, M.; Damin, F.; Longhi, R.; Gotti, C.; Galati, C.; Renna, L.; Chiari, M. Peptide microarrays on coated silicon slides for highly sensitive antibody detection. *Methods Mol. Biol.* **2010**, *669*, 147–60.
16. McMurry, J. A.; Kimball, S.; Lee, J. H.; Rivera, D.; Martin, W.; Weiner, D. B.; Kutzler, M.; Sherman, D. R.; Kornfeld, H.; De Groot, A. S. Epitope-driven TB vaccine development: a streamlined approach using immuno-informatics, ELISpot assays, and HLA transgenic mice. *Curr. Mol. Med.* **2007**, *7* (4), 351–68.

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

17. Vigil, A.; Chen, C.; Jain, A.; Nakajima-Sasaki, R.; Jasinskas, A.; Pablo, J.; Hendrix, L. R.; Samuel, J. E.; Felgner, P. L. Profiling the humoral immune response of acute and chronic Q fever by protein microarray. *Mol. Cell Proteomics* **2011**.
18. Lee, W. L.; Li, J.; Uttamchandani, M.; Sun, H.; Yao, S. Q. Inhibitor fingerprinting of metalloproteases using microplate and microarray platforms: an enabling technology in Catalomics. *Nat. Protoc.* **2007**, *2* (9), 2126–38.

Downloaded by PENNSYLVANIA STATE UNIV on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch009

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

Chapter 10

pH-Directed Self-Assembling Helical Peptide Conformation

J. Vincent Edwards,* Alfred D. French, Thomas Jacks,[1] and K. Rajasekaran

Southern Regional Research Center, ARS-USDA, 1100 Robert E. Lee Blvd., New Orleans, Louisiana 70124
***E-mail: vince.edwards@ars.usda.gov**
[1]Posthumous author.

The beta-sheet and alpha-helix peptide conformation are two of the most fundamentally ordered secondary structures found in proteins and peptides. They also give rise to self-assembling motifs that form macromolecular channels and nanostructures. Through design these conformations can yield enhanced membrane activity. The self-assembling properties of the beta-sheet and helical peptide motifs have found many applications as antimicrobials and in biomaterials with potential in regenerative medicine. In a delivery or biomaterial system these two conformational motifs can confer nano-strucutral properties that are useful in implantable biomaterials, and non-viral gene formulations. Influenza hemagglutinin (HA) fusion peptides, which were first reported by Wiley et al., possess lytic properties of HA that allow it to gain endosome entry through conformational transition, and is required for membrane fusion activity. The helical peptide's active conformation is formed by way of a pH-triggered change in conformation that is endosomolytic as originally found in HA mutants. Here we discuss helix design properties of some pH-triggered lytic peptides while maintaining a conformation with minimum amino acid chain length requirements.

Not subject to U.S. Copyright. Published 2012 by American Chemical Society
In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

Introduction

The beta-sheet, helix and reverse turn conformations of peptide design have been historically employed in neuropeptides (*1–3*) and pharmaceutically active peptide analogs (*4*, *5*). The participation of peptide conformation in design properties is inclusive of virtually most therapeutic disease categories including but not limited to lung surfactants (*6*, *7*), antimicrobials (*8*, *9*), growth factor agonists and antagonists (*10*, *11*), and enzyme recognition (*12*) to name a few examples that our associated laboratories have explored. Since the first biosynthetic insulin became available in 1982 the important role of secondary structural conformation has been essential in designing deliverable, bio-available analogs of insulin used by millions of diabetics (*13*). The discernment of the relation of peptide conformational motifs to concomitant bioactive properties has recently been of interest in extracorporeal and implantable biomaterials as bio-conjugates in dressings (*12*, *14*, *15*), therapeutic gene formulations (*16*, *17*), self-assembled nonwoven biomaterials for wound healing and tissue engineering (*18*, *19*), and combined gene therapy and tissue engineering (*20*).

The use of non-viral peptide-based gene delivery systems to effect gene delivery and cell nucleus transfer poses a promising advantage over viral gene delivery (*21*, *22*). Rationally designed peptide-based gene delivery systems provide a flexible, safe, approach to potential therapeutic gene administration (*23*, *24*). Self assembling DNA complexes that consist of a cationic DNA-condensing peptide and an anionic endosomolytic peptide have been reviewed (*25*). In the course of being endocytosed by the cell membrane the complex is entrapped in a low-pH endosome and may be released only through lysis of the membrane that forms the endosome compartment of the cell. The endosomolytic peptides viral entry into a cell occurs through membrane lysis based on specific peptide sequences in the viral coat proteins. Wagner et al. showed how peptide sequences forming the lytic portion of these viral coat proteins could be used to effect endosomal release (*26*). Both Szoka and Gottschalk et al. have further demonstrated de novo optimization of the gene deliviery systems containing rationally designed lytic peptides (*22*, *27*). The design and synthesis of peptides having selectively pH-sensitive lytic properties of helical peptides has previously been reported based on amphipathic membrane associating properties of helical peptides (*25*). Helical endosomolytic peptides contain a hydrophobic face possessing non-polar residues and a hydrophilic face possessing negatively charged residues. Peptides of this nature are known for their endosomal release properties, due to their propensity to form a alpha-helical secondary structure at the acidic pH of the endosome. Both Wiley and Degrado have reported the HA2 peptides of the influenza hemagglutinin and their fusion properties (*27–29*). We desired to minimize the sequence requirements of the endosomolytic peptide of the self-assembling DNA complex while retaining its pH sensitive lytic properties to optimize the structure function properties of the complex as a peptide-based delivery system. Here we report the pH-dependent change in conformation of small helical peptides, and their selective activity.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

Table 1. Peptide Sequences of five fusogenic helical peptides and one beta-sheet peptide given in single letter abbreviation. Suc = succinyl acylating the amino terminus and # represents an amide group at the COOH-termini. Lytic activities reported as Lytic Concentrations at 50% LC50 (mg/mL), maximum percent lysis of erythrocytes, and the concentration at maximum lysis (mg/mL), as reported in the Materials and Methods section

Peptide amino acid sequence	*Hemolytic Selectivity*	*LC_{50}*	*MAX. % Lysis*	*Concentration at Maximum Lysis*
SucLLEK-LLEEWLE# (Selective) I	Selective	0.0071	75.2	0.138
SucLLEK-LLEWLE# (Nonselective) II	Nonselective	0.00529	100	0.109
GLFEKLKEWLE# (Nonselective) III	Nonselective	0.019	95.8	0.0401
GLFKELWKELE# (Selective) IV	Selective	0.7306	64.9	0.94
GLFKEALEEL-WEA# (Selective) V	Selective	0.102	131.8	0.266

Materials and Methods

Peptide Synthesis, Characterization, Conformation, and Modeling

The peptides were synthesized on CLEAR (obtained from Peptides International, Louisville KY). Amino-functionalized CLEAR- amide resin, 100-200 mesh was employed in the synthesis. The synthetic protocol for the synthesis of conjugate I on CLEAR resin consisted of the following steps. The resin was washed 3 × with 20 mL of dimethylformamide (DMF) (5 min) and 3 × with 20 mL of dichloromethane (DCM) (5 min). 9-flourenylmethyloxycarbonyl-AA-CLEAR (FMOC-AA-NHC(O)CLEAR) resin was deprotected in 20% piperdine/DMF by shaking the mixture 3 times in 15-min intervals. Fmoc amino acids were consecutively coupled with diisopropylcarbodiimide (DIC) and 1-hydroxy-7-azabenzotriazole (HOBT) in DMF for 120 minutes. Both the deprotection and the reaction coupling cylces were monitored with the Kaiser test. All peptides were analyzed for the appropriate amino acid content and subject to FAB MS where they were identified with the appropriate [M + 1] parent ion as well.

The circular dichroism spectra were measured on a Jasco J-500A spectropolarimeter performed by Dr. Thomas Jacks and hereby posthumously entered. The molecular modeling program Chem-X, no longer distributed, was used to generate the six peptides as alpha helices having amino acids with standard geometries and the pore models shown in Figures 2 and 3.

Erythrocyte Lysis Assay

Freshly prepared human erythrocytes were washed with HBS and resuspended in a 2 x assay buffer of the appropriate pH (300 mM NaCl, 30mM sodium citrate) at a concentration of 7 x 107/mL. An aliquot of 75 microliters was added to 75 microliters of a serial dilution of the peptide in a 96-well microtiter plate and incubated for 1h at 37°C with constant shaking. After removal of the unlysed erythrocytes by centrifugation (1000 x g, 5 min), 100 microliters of the supernatant were transferred to a new microtiter plate, and hemoglobin absorption was measured at 450 nm. 100 percent lysis determined by adding 1 microliter of a 10% Triton X-100 solution prior to centrifugation. The hemolytic units were calculated as the receiprocal valute of the peptide concentration, where 50% leakage was observed.

Results and Discussion

The peptides of this study were selected based on past fusion peptide studies that have their origin in Wiley et al.'s pioneering work on influenza hemagglutinin fusion peptides (*30*, *31*) and subsequent studies conducted on endosomolytic peptides (*32*). The peptides shown in Table 1 were designed as portrayed in Figure 1 to adopt an amphipathic alpha helix. Previous studies have shown that the minimal length of a helical peptide for interaction with phosphatidylcholine liposomes is eighteen residues (*7*). However, the peptides of this study were minimized to 10 – 13 residues, and their lytic activity suggests effective interaction with cell wall lipids. Based on Chou-Fasman rules (*34*) helical wheel designs of amphipathic helices were made to create a hydrophilic face and hydrophobic face wherein intra-molecular hydrogen bonding was optimized including the addition of both N-terminal succinylation and the COOH-terminal amide.

Peptide I possessed selective lytic activity and contains principally α-helix forming glutamate and leucine residues with a single lysine and tryptophan residue. As seen in Figure 2 the CD spectrum for Peptide I at acidic pH demonstrated a spectrum characteristic of an α-helical conformation in a strong hydrogen-bonding environment (*33*). This spectrum has a negative band starting at 230 nm due to the nπ* transition (promotion of a nonbonding orbital on the carbony oxygen to the antibonding π* orbital of the amide group) and an amide bond exciton splitting of the 190-nm ππ*, which gives rise to the negative band at 208 nm and the positive band at 192 nm. On the other hand the CD of Peptide I at neutral pH is characterized by a single deep minimum near 200 nm which is characteristic of the ππ* transition and appears only in peptides with chain lengths

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

of 10 or more residues. This spectrum is also characterized by the absence of an absorption minimum corresponding to the nπ* transition.

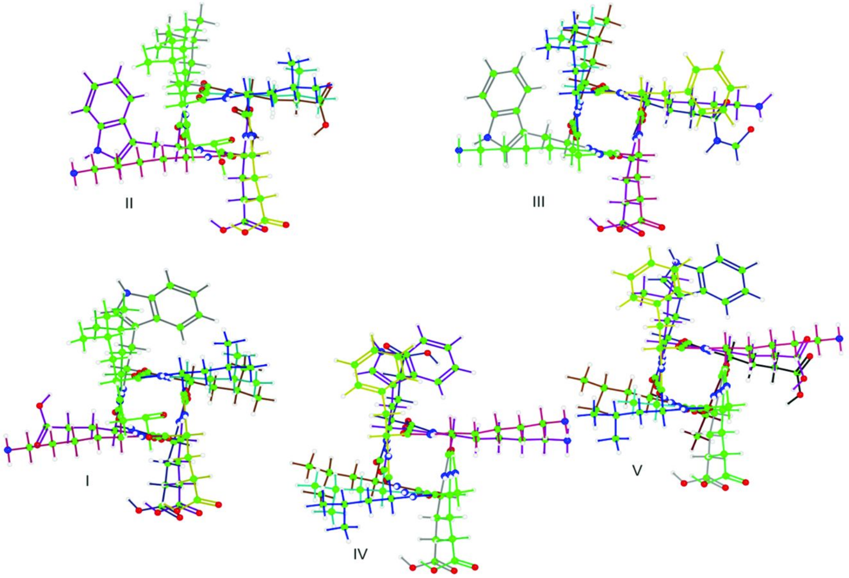


Figure 1. Helical wheel structures of Peptide I – V. The top two structures Peptides II and III have lytic activity only at acidic pH (pH 4). Whereas the bottom three have lytic activity at pH 4 and 7. (see color insert)

Peptide II is a nearly identical amino acid sequence to Peptide I with the exception of deletion of the glutamate N-terminal to tryptophan in Peptide I to give a 10 residue sequence. This analog demonstrated a non-selective lytic activity. Thus, the additional glutamate residue in Peptide I at this position is necessary for selective lytic activity. As seen by the CD of Peptide II the spectrum at acidic pH gave the highly distinctive minima at 208 nm and 222 nm, characteristic of the α-helix (*25*). Interestingly the conformation of Peptide III is identical to Peptide II at both acidic and neutral pH.

The non-selective activity versus selective activity is also seen with the 11-mer motif design in Peptide III and IV. The structure of Peptide IV shares some homology with Peptide III as well as varies at the COOH-terminus and in placement of acidic and basic amino acids. With Peptide IV selective lytic activity is achieved by substituting a Lys-Glu at the fourth N-terminal residue for Glu-Lys and rearranging the Leu-Lys-Glu-Trp segment in Peptide III to Leu-Trp-Lys-Glu. The net secondary structural effects of these substitutions that give rise to selective lytic activity at acidic pH are seen in Figure 1. Since both of these peptides share structural homology throughout the chain length it is interesting to speculate on the structural basis of the different lytic activities. As discussed below the principle design feature in pH-triggered lytic activity appears to be a well defined separation of the hydrophilic and hydrophobic faces.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

Figure 2a: Peptide I

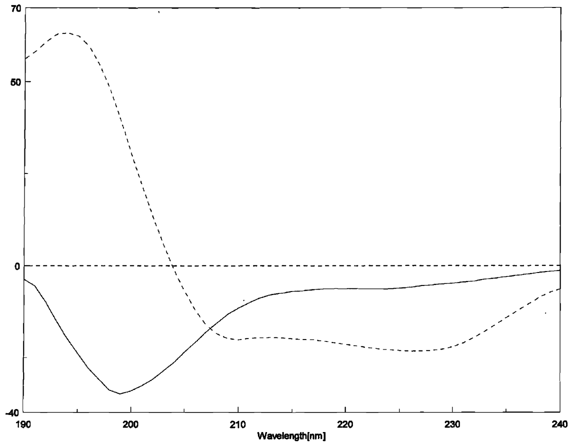


Figure 2b: Peptide II

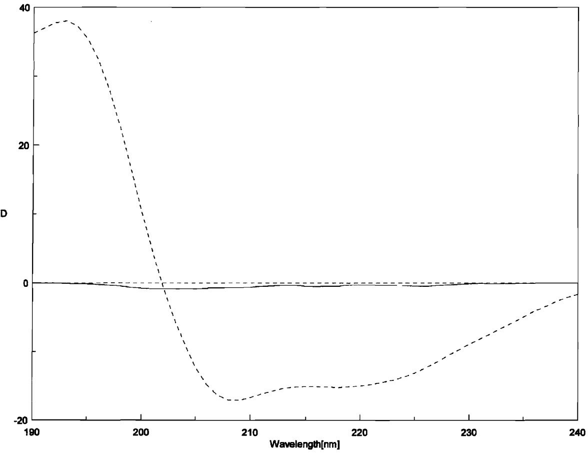


Figure 2c: Peptide III

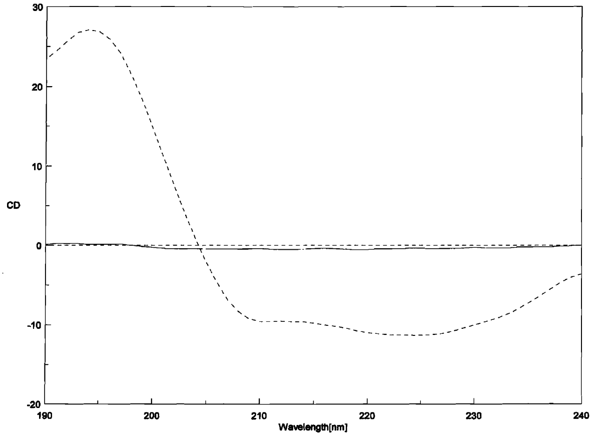


Figure 2d: Peptide IV

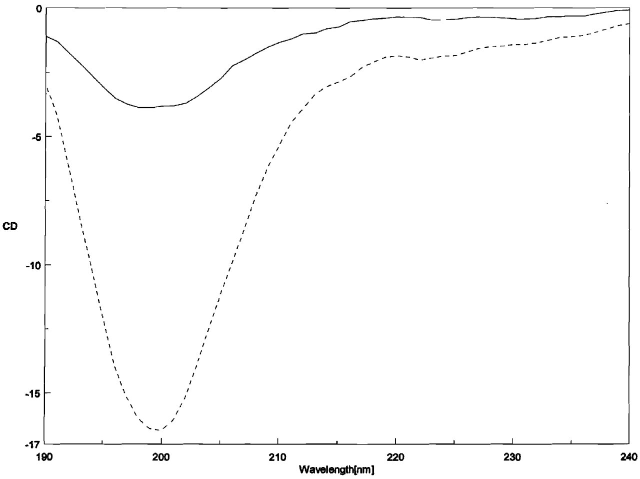


Figure 2e: Peptide V

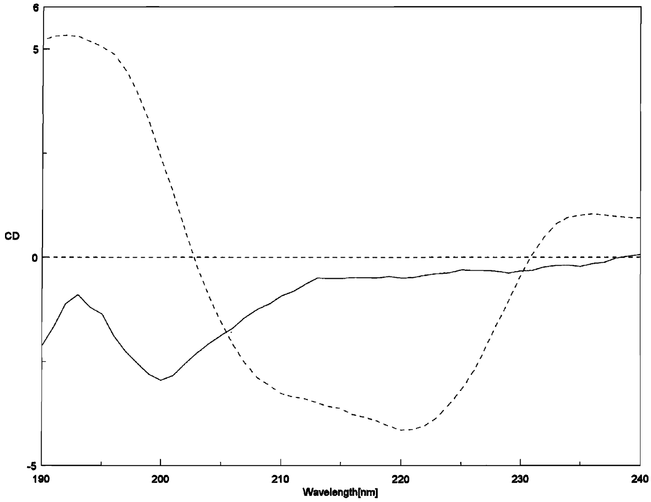


Figure 2. CD spectra of Peptides I – V (2a–e, consecutively I-V). The dashed lines represent spectra taken at acidic pH (pH 4), and the solid lines represent spectra taken at neutral pH (pH 7).

The observed effect of the selectively lytic peptide amino acid variations on the helical wheel design is as follows; clear cut separation of the hydrophilic from hydrophobic face i.e. all hydrophilic and hydrophobic residues are co-resident within the four residues of each helical turn and are well separated in the helical wheel, close proximity of a lysine side chain amino to glutamate side chain carboxylate to form a salt bridge in one side of the helical wheel, and co-residence of adjacent glutamate side chains within the other side of the wheel. These are all structural features shared by the three selectively lytic analogs. Whereas, Peptide analogs II and III, which are non-selectively lytic, share overlap of the

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

hydrophilic and hydrophobic faces. An example of this is readily observed with the lysine and tryptophan side chains, which are 7.3 angstroms (from side chain amine to indole ring nitrogen) apart in both analogs. Lysine and tryptophan are separated by three residues within the peptide chain, and co-resident within one corner of the helical wheel.

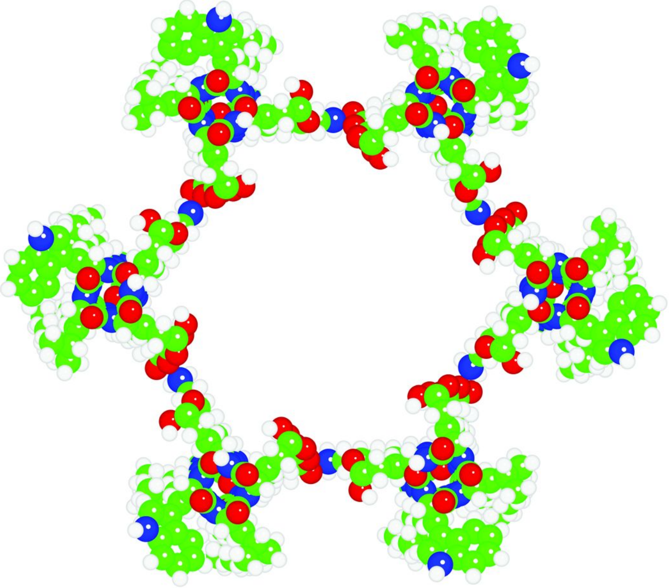


Figure 3. Illustration of a putative pore structure formed from Peptide I with hydrophilic residues directed inward and hydrophobic residues directed outward to promote interaction with membrane lipids. (see color insert)

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

It can be inferred from the results of the lytic activity and the CD observed conformations that Peptides II and III probably interact differently with membrane bilayers in contrast with Peptides I, III, and IV. The mechanism of membrane-active amphipathic helixes has been reviewed extensively ((*35*),38), and can be understood in terms of the concept of membrane monolayer curvature strain. In a membrane which is composed of two apposing monolayers there is no tendency for the membrane bilayer to curve. The binding of an amphipathic helical peptide (AHP) to a lipid bilayer will often promote either positive or negative membrane curvature. For example the protein plasma apolipoprotein A-I and model peptides that mimic its amphipathic helical portions (class A helices) can stabilize membranes giving less hemolysis and a variety of other beneficial effects including the ability of high density lipoprotein (HDL) to protect against atherosclerosis (*35*). It is thought that stabilization of membranes by class A peptides occurs by increasing the intrinsic radius of curvature of the membrane. On the other hand as seen in this study, AHPs can induce lysis by pore formation, and possibly any peptide can at a high enough peptide to lipid ratio, which results in ion leakage and cell lysis. However, certain peptides form pores at relatively low mole fractions in the membrane. An example of this is seen in lytic peptides similar to the ones of this study, and in helix-2 of the Bacillus thuringiensis israelensis toxin. These type of pore forming peptides can be lytic to cell membranes through causal leakage of ions through the membrane (*36*). Figure 3 illustrates how a putative pore constructed of Peptide I might form from a hexamer resulting from ion-pairing of lysine and glutamate residue side chain amino and a carboxylate groups such that the hydrophobic residues are facing outward and the hydrophilic residues inward. This type of ion pairing would occur at acidic pH, and could explain the selective lytic activity of Peptides I, IV, and V. Antimicrobial peptides have been studied extensively for their pore forming mode of action (*37*).

This study has examined five peptide analogs for their self-assembling properties as relatively small helical peptides and the relation of their conformation to lytic activity. Distinctive structure function relations delineated here may be useful in the design of cell entering peptides with specific membrane active properties as has recently been reported (*21*). In particular the overall relation of acidic and basic amino acids placed in the helical wheel, and formation of a prominent hydrophobic and hydrophilic face is important to conformation and membrane binding. The most potent lytic analog Peptide II was also the shortest sequence demonstrating that optimal design is as important as the total number of residues employed to achieve selective lytic activity.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

References

1. Taylor, J. W.; Kaiser, E. T. *Pharmacol. Rev.* **1986**, *38* (4), 291.
2. Hughes, J.; Smith, T. W.; Kosterlitz, H. W. *Nature* **1975**, *2* (58), 577–579.
3. Schiller, P. W.; Dimaio, J. *Nature* **1982**, *279*, 74–76.
4. Roques, B. P. *J. Peptide Sci.* **2001**, *7*, 63–73.
5. Stahl, M.; Guba, W.; Kansy, M. *Drug Discovery Today* **2006**, *11* (7-8), 326–333.
6. McLean, L. R., Edwards, J. V. U.S. Patent 5,861,481, 1999.
7. McLean, L. R.; Hagaman, K. A.; Owen, T. J.; Krstenansky, J. L. *Biochemistry* **1991**, *30*, 31–37.
8. Jenssen, H. *Exp. Opin. Drug Discovery* **2011**, *6* (2), 171–184.
9. Rajasekaran, K.; Stombery, K. D.; Cary, J. W.; Cleveland, T. E. *J. Agric. Food Chem.* **2001**, *49*, 2799–2803.
10. Edwards, J. V.; McLean, L. R.; Wade, A. C.; Eaton, S. R.; Cashman, E. A.; Hagaman, K. A.; Fanger, B. O. *Int. J. Peptide Protein Res.* **1994**, *43*, 374–383.
11. Malikayil, J. A.; Edwards, J. V.; McLean, L. R. *Biochemistry* **1992**, *31*, 7043–7049.
12. Edwards, J. V.; Batiste, S. L.; Gibbins, E. M.; Goheen, S. C. *J. Peptide Res.* **1999**, *54*, 536–543.

13. Brange, J.; Volund, Aage *Adv. Drug Delivery Rev.* **1999**, *35*, 307–335.
14. Hashimoto, T.; Suzuki, Y.; Tanihara, M.; Kakimaru, Y.; Suzuki, K. *Biomaterials* **2004**, *25* (7-8), 1407–1414.
15. Mertz, P. M.; davis, S. C; Franzen, L.; Uchima, F.-D.; Pickett, M. P.; Pierscbacher, M. D.; Polarek, J. W. *J. Burn Care Rehabil.* **1996**, *17* (3), 199–206.
16. Branski, L. K.; Pereira, C. T.; Herndon, D. N.; Jeschke, M. G. *Gene Ther.* **2007**, *14*, 1–10.
17. Guo, R.; Xu, S.; Ma, L.; Huang, A.; Gao, C. *Biomaterials* **2011**, *32*, 1019–1031.
18. Gelain, F. *Int. J. Nanomed.* **2008**, *3* (4), 415–424.
19. Ma, X. P. *Adv. Drug Delivery Rev.* **2008**, *60*, 184–198.
20. Coutu, D. L.; Yousefi, A.-M.; Galipeau, J. *J. Cell. Biochem.* **2009**, *108*, 537–546.
21. Lehto, T.; Simonson, O. E.; Mager, I.; Ezzat, K.; Sork, H.; Copolovici, D.-M.; Viola, J. R.; Zaghloul, E. M.; Lundin, P; Moreno, P.MD; Mae, M.; Osholkov, N.; Suhorutsenko, J.; Smith, C. E.; Andaloussi, S. E. *Mol. Ther.* **2011**, *19* (8), 1457–1467.
22. Legendre, J.-Y.; Szoka, F. C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 893–897.
23. Gottschalk, S.; Sparrow, J. T.; Hauer, J.; Mims, M. P.; Leland, F. E.; Woo, S. L.; Smith, L. C. *Gene Ther.* **1996**May, *3* (5), 448–57.
24. Wilke, M.; Fortunate, E.; Broek, M. vd.; Hoogeveen, A. T.; Scholte, B. J. *Gene Ther.* **1996**, *3*, 1133–1142.
25. Smith, L. C.; Duguid, J.; Wadhwa, M. S.; Logan, M. J.; Tung, C.-H.; Edwards, V.; Sparrow, J. T. *Adv. Drug Delivery Rev.* **1998**, *30*, 115−131.
26. Plank, C.; Oberhauser, B.; Mechtler, K.; Koch, C.; Wagner, E. *J. Biol. Chem.* **1994**, *289* (17), 12918–12924.
27. Steinhauer, D. A.; Martin, J.; Lin, Y. P.; Wharton, S. A.; Oldstone, M. B. A.; Skehel, J. J.; Wiley, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12873–12878.
28. Skehel, J. J.; Bizebard, T.; Bullough, P. A.; Houghson, F. M.; Knossow, M.; Steinhauer, D. A.; Sharton, S. A.; Wiley, D. C. *Cold Spring Harbor Symposia on Quantitative Biology*; Cold Spring Harbor Laboratory Press: New York, 1995; Vol. LX, pp 573−580.
29. Lau, W. L.; Ege, D. S.; Lear, J. D.; Hammer, D. A.; Degrado, W. F. *Biophys. J.* **2004**, *86*, 272–284.
30. Wilson, I. A.; Skehel, J. J.; Wiley, D. C. *Nature* **1981**, *289*, 366–373.
31. Wiley, D. C.; Wilson, I. A.; Skehel, J. J. *Nature* **1981**, *289* (5796), 373–8.
32. Steinhaure, D. A.; Martin, J.; Lin, Y. P.; Stephen *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12873–12878.
33. Woody, R. W. Circular dichroism of peptides. In *Conformation in Biology and Drug Design*; Udenfriend, S., Meienhofer, J., Eds.; Academic Press, Inc.: Orlando, FL, 1985; Chapter 2, pp 16–104.
34. Chou, P. Y.; Fasman, G. D. *Biochemistry* **1974**, *13*, 211–222.
35. Epand, R. M.; Shai, Y.; Segrest, J. P.; Anatharamaiah, G. M. *Biopolymers* **1995**, *37*, 319–338.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

36. Maddrell, S. H. P.; Overton, J. A.; Ellar, D. J.; Knowles, B. H. *J. Cell Sci.* **1989**, *94*, 601–608.
37. Sakai, N; Matile, S. *Chem. Commun.*, 2514–2523.

Downloaded by OHIO STATE UNIV LIBRARIES on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch010

Chapter 11

Controlling Symbiotic Microbes with Antimicrobial Peptides

Peter Mergaert*,[1] and Eva Kondorosi[1,2]

[1]Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France
[2]Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary
***E-mail: peter.mergaert@isv.cnrs-gif.fr**

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

The production of antimicrobial peptides (AMPs) in response to invading pathogenic microbes is an effective and ancient innate immune strategy which is conserved in all analyzed present-day eukaryotes. However, organisms are not only threatened by microbes but on the contrary, they often form beneficial symbiotic associations with them. There is a growing number of reported cases, both in animals and plants, which demonstrate the critical involvement of AMPs also in these symbiotic interactions. Notably, AMPs can intervene in the selection and maintenance of symbiotic microbial communities.

Acquiring New Capabilities through Symbiotic Associations

Multicellular organisms live not free of germs but are inhabited with a multitude of microbes. This microbiota includes symbionts which co-evolved with their hosts for millions to billions of years. They contribute significantly to the hosts normal development and growth in a wide range of means. In this way, the microbial symbionts endow the hosts with capacities that they did not need to evolve on their own.

The animal gut is crowded with symbiotic microbes. For example, the human gut has an estimated load of 10 to 100 trillion bacteria representing from several hundreds to thousands of species (*1*). These gut symbionts have a profound impact on gut development. They direct the proliferation and maturation of gut epithelial cells for epithelium renewal in drosophila (*2*), zebrafish (*3*, *4*) and

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

mammals (*5*). Gut bacteria in mice have been shown to stimulate intestinal blood vessel development (*6*). The mammalian gut symbionts also shape the intestinal and systemic immune system by coordinating the differentiation of pro-inflammatory and anti-inflammatory T cells (*7–10*).

Other well documented symbioses involve organ development and often require an active participation of the microsymbiont in the organogenesis. The marine squid *Euprymna scolopes* for example has a light-emitting organ which is colonized by luminescent *Vibrio fischeri* bacteria. This light organ helps the animal in evading predators. The sequential steps in the colonization and tissue differentiation of the light organ are coordinated by bacterial signals (*11*). Aphids and other insects harbor intracellular symbionts in specialized cells called bacteriocytes which are organized in the bacteriome organ located on the abdomen of the insect (*12*). Another well described system for symbiotic organ development is the symbiosis of legume plants with soil bacteria belonging to the *Rhizobiaceae* (collectively called rhizobium bacteria or rhizobia). This interaction leads to the formation of a dedicated organ, the nodule. Nodules develop on the root system of the host, and house the intracellular symbiotic bacteria. The nodule formation entirely depends on the presence of rhizobia and is induced by a specific bacterial signal called the Nod factor (*13*).

The raison d'être of many symbionts is to provide the ability to harvest otherwise inaccessible nutrients. Gut microbes form an anaerobic bioreactor which hydrolyze ingested polysaccharides and ferments the resulting monosaccharides. The by-products of fermentation are short chain fatty acids which are absorbed by the host and utilized as carbon and energy source (*14*, *15*). Aphids feed exclusively on plant phloem sap which constitutes a diet that is deficient in amino acids. The pea aphid *Acyrthosphion pisum* harbors the obligate endosymbiont *Buchnera aphidicola* in its bacteriocytes which synthesizes the essential amino acids missing in the phloem and provides them to the host (*16*). Likewise, the endosymbiotic rhizobium bacteria in legume nodules fix air-borne nitrogen gas, an abundant but stable chemical form of nitrogen, unusable for most organisms except nitrogen-fixing microbes. The ammonium resulting from the nitrogen reduction by the nodule rhizobia is assimilated by the host plant and used for its growth (*17*).

Hosts Actively Select and Control Their Microbial Symbionts

An important issue in symbiosis is to pick out the right bacterial partner, often among a myriad of environmental microbes. Hosts are able to select actively their microbiota. In addition, once the host is colonized by the right symbionts, mechanisms must be put in place to ensure that the association is stable, that the symbionts do not overgrow the host or on the contrary, that the host does not eliminate the symbionts. Effective symbiont selection can happen through the vertical transmission of the symbionts from parent to offspring through the female germ line as it happens for example in the aphid-*Buchnera* symbiosis (*16*). But even microbes that are transmitted horizontally are successfully selected from the environment. This may rely on complex mechanisms implying developmental

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

processes which are induced by bacterial signals. Such mechanisms in squid and legumes are exquisitely specific, capable to select a single bacterial species from the extremely complex microbiota of the environment, the sea water or the rhizoshere soil respectively (*18*).

The specific colonization of the squid light organ by *V. fischeri* for example is ensured by a complex series of developmental responses, including gathering of bacteria from the seawater by moving cilia, production of mucus that specifically aggregates *V. fischeri*, recruitment of haemocytes, cell differentiation and apoptosis. Some of these events are induced by bacterial peptidoglycan and lipopolysaccharide signals and require specific *V. fischeri* genetic determinants such as the two-component regulator RscS/SypG which controls the production of the Syp (Symbiosis polysaccharide) exopolysaccharide mediator of colonization (*11*, *19–21*). Established *V. fischeri* populations in the light organ are kept in check by the daily expulsion of most of the bacterial population each dawn, followed by the regrowth of the remaining bacteria (*22*). In the case of the legume root nodules, the rhizobium-produced Nod factors induce the formation of the nodules and also the formation of tubular infection structures called infection threads which guide the rhizobia inside the plant tissues but exclude penetration of any other bacteria into the nodule tissues (*13*). Rhizobia are released massively from infection threads in the target nodule cells and differentiate into nitrogen fixing "bacteroids". In some legumes, the host cells control the intracellular bacterial population by the production of AMPs which induce an irreversible, terminally differentiated state of the bacteroids (*23*).

Also in the seemingly "open" system of the animal gut where bacteria are acquired by oral uptake, the symbionts are actively selected by the host. The human intestine contains, compared to the 55 known bacterial divisions (deep evolutionary lineages), few divisions and is dominated by only two of them, the Firmicutes and the Bacteroidetes. This contrasts with soils where plant polysaccharides are also degraded, containing at least twenty bacterial divisions and suggests that the human gut microbiota are at least in part selected by the gut epithelium (*24*). This is corroborated by studies of the bacterial diversity in the guts of 60 mammalian species including humans (*25*) or of wild great apes (*26*), both indicating that the microbial communities co-diversified with the host phylogeny. When gut microbiotas from mouse and zebrafish were reciprocally transplanted in germ-free zebrafish and mouse hosts respectively, the transplanted microbial communities were transformed to resemble the normal microbiota composition of the recipient host, thus revealing again an active selection by the host (*27*).

The complexity of the gut microbiota is highly different among animals and the bacterial diversity in the studied invertebrates is apparently one to two orders of magnitude lower than in the mammals. Nevertheless, in each case this bacterial population has a specific composition. For example, the Drosophila gut is dominated by just a few dominant bacterial species (*28*). Even in Cnidaria, the simplest animals positioned in the earliest branches of the animal tree of life, the epithelial cells actively shape their bacterial community (*29*).

Moreover, the symbiotic bacteria in animal guts are numerous and thus they pose a threat of invasion. Therefore, the animal host has to build the specific

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

gut microbiota and maintain it in a homeostatic relationship with the epithelial cells that are in contact with it. Then, how is this achieved? In mammalians, specific host factors that determine the composition of the gut microbiota and keep them in equilibrium are the innate and adaptive immune systems (*30*, *31*) with a primary role for AMPs (*32*). But also in Drosophila and Cnidaria, AMPs are key factors that influence the structure of the gut microbial community (*28*, *33*). Thus, host AMPs are conserved metazoan key actors in the interaction with epithelial microbiota.

AMPs: Fighting Pathogens and Controlling Symbionts

AMPs are important players in plant and animal innate immune systems. AMPs are extremely diverse, differing in their amino acid composition and structure. They affect immunity in broadly two ways. They can kill microbes and thus act as antibiotics displaying usually fast and broad spectrum killing activities against Gram-negative and Gram-positive bacteria, fungi as well as viruses and parasites (*34*). In addition, AMPs may enhance immunity by functioning as immune-modulators (*35*). AMPs are best known for this role in innate immunity, in fending off microbes from the environment and in fighting pathogenic infections. However, as underlined above, recent findings in different organisms including invertebrates, vertebrates and plants have demonstrated the unexpected importance of AMPs in the selection of bacterial symbionts and the control of established symbiotic populations. These new insights in symbiotic mechanisms and the natural roles of AMPs in symbiosis will be reviewed here. The examples in the literature of AMPs controlling gut microbiota will be briefly highlighted and then the focus will be on specific AMPs that control the endosymbiotic rhizobium bacteria in legume nodules.

Control of the Animal Gut Microbiota by AMPs

Colonization of Epithelia in the Early Branching Metazoan Hydra

The cnidarian Hydra (polyps) maintain a specific bacterial microbiota as indicated by comparing laboratory populations of different species and specimens directly isolated from the wild. Different species grown under identical laboratorium conditions over more than thirty years differed greatly in their microbiota but specimens living in the wild were colonized with a similar microbiota as the laboratory polyps of the same species (*29*). Hydra have a simple tube-like body enclosing the digestive cavity with at one end a mouth and tentacles. This body is enclosed by an equally simple tissue organization consisting of two single-cell layers, the outside ectoderm and the inside endoderm. In between these layers are interstitial cells which are stem cells that can give rise to various cell types including the germ line, nerve cells, gland cells and nematocytes (the venomous nettle cells on the tentacles, the distinguishing feature of Cnidaria). Genetic ablation of the interstitial cell lineage in the temperature-sensitive mutant strain sf1 of the species *Hydra magnipapillata* changes the microbiota of the animals considerably. In particular, the dominant β-proteobacteria phylotype

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

in the control animals is reduced in the interstitial-cell-line-depleted animals, and on the contrary, the Bacteroidetes phylotype becomes more abundant (*36*). These results indicate that the ablated cells produce factors that interfere with the microbiota and control its structure. Purification of antimicrobial activities in Hydra protein extracts as well as screening for differentially expressed genes has led to the identification of several AMP families and other antimicrobial proteins produced by the Hydra epithelial and interstitial cells (*33*, *37–40*). The impact on the microbiota of one of those AMPs named periculin 1a was analyzed in more detail (*33*). It was noticed that the composition of the microbiota in early embryos was very different from those at later embryonic stages and adult animals. Thus the early embryos produce specific factors controlling the bacterial colonizers. One of these factors was biochemically identified as the AMP periculin 1a. This peptide is specifically expressed in a subset of the interstitial cells of the female germ line, in the developing oocytes and the early embryos (*33*). When the periculin 1a peptide was ectopically expressed in the ectodermal cell layer of adult polyps, the bacterial load in the animals was strongly reduced and interestingly, also the specific composition of the microbiota was dramatically changed with a strong decrease of the dominant β-proteobacteria, an equally strong increase of the α-proteobacteria and the appearance of new phylotypes (*33*). Together, these findings make a strong case for a role of AMPs in the selection of particular bacterial symbionts during the animal development and adult life in Hydra.

The Drosophila Gut

Among the major immune reactions of Drosophila in response to microbial infections is the inducible production of AMPs (*41*). AMPs are induced during the so-called systemic response in the fat body and secreted in the hemolymph circulatory system of the insect. Moreover, they are also induced locally in epithelial cells in a tissue-specific manner. Induction of the AMPs in the gut is mediated by the immune deficiency (Imd) pathway. The pathway is activated by the peptidoglycan recognition proteins which bind the peptidoglycan component of the cell envelop of gram-negative bacteria. This subsequently results through an intracellular signalling pathway in the proteolytic activation and nuclear translocation of the NF-κB transcription factor Relish, followed by the transcription of AMP genes (*41*). Thus, oral infection with bacterial pathogens will lead to AMP activation in the gut and clearance of the pathogen. However, this leaves open the question how the homeostasis between this innate immune response and the symbiotic microbiota is achieved.

Surprisingly, it was found that the resident bacteria in the Drosophila gut chronically activate the Imd pathway as revealed by the nuclear localization of Relish in conventionally reared flies but not in germ-free flies. Yet, this does not activate AMP gene transcription (*28*). The reason is the repression of AMP expression by the homeobox transcription factor Caudal (*28*), well known for its role in embryo formation and development of the gastrointestinal tract (*42*) and for constitutive AMP expression in certain epithelial cells (*43*). Inactivation of Caudal via RNA interference (RNAi) provoked a spontaneous activation of AMP gene expression in the gut which was however dependent on the presence of the microbiata and did not take place in germ-free flies. Thus AMP expression in the gut epithelia is the result of a balance between activation by Relish and repression by Caudal (*28*).

Inactivation of Caudal by RNAi provoked along with the high production of AMPs, also a dramatic shift in the microbiota community (*28*). In particular, the abundance of a dominant *Acetobacter* species in control flies was greatly reduced by Caudal RNAi while in contrast, a minor *Gluconobacter* species of the wild type gut emerged as a dominant one. Still other gut bacteria were not affected by the Caudal inactivation. Ectopic AMP expression in transgenic flies resulted in the same changes in the gut bacterial community. Moreover, the sensitivity of these bacteria to the *in vitro* applied, pure AMP correlated with the *in vivo* observations. Thus, the observed modifications in the microbiota are the direct consequence of deregulated AMP expression (*28*).

Additionally, the dysbiosis (microbial imbalance) resulting from the dominance of the *Gluconobacter* species in the gut of the AMP overexpressing flies led eventually to the rupture of the gut homeostasis and induced apoptosis of intestinal cells and host mortality (*28*). Apoptosis and mortality were high in conventionally reared Caudal RNAi flies but not in germ free flies and simulation of the dysbiosis by introducing the *Gluconobacter* strain in germ free wild type flies induced the same. Thus the dysbiosis is the direct cause of the gut pathology. All together, this study shows that a controlled and balanced expression level of AMPs under healthy conditions is essential for the maintenance of the normal gut flora and the microbial composition is on its turn important for the homeostasis between the microbiota and the innate immunity in the gut of flies.

The Mammalian Gut

Maintaining intestinal homeostasis in mammalians and particularly in humans has another level of complexity in light of the astonishing quantity and species diversity of their gut microbiota. Thus, it is a challenging task for the intestinal epithelia to prevent that these microbiota penetrate in the underlying tissues. This is achieved by a multitude of immunological barriers which are designed to minimize the direct contact of the epithelia with the intestinal bacteria and to rapidly eliminate bacteria that penetrate anyhow the epithelia and the underlying lamina propria tissue (reviewed in (*30*)). The latter occurs through phagocytosis by macrophages and T cell-mediated responses. The barrier which confines the microbiota essentially to the gut lumen is composed of three key elements (*30*). First, a protective mucus layer composed of mucin glycoproteins is produced and secreted by the goblet cells, a specific epithelial cell type. This layer creates a nearly bacteria-free zone on the surface of the epithelia which is lost in mutant mice lacking the major mucin protein MUC2 (*44*, *45*). Second, immunoglobulin A (IgA), specific for intestinal bacteria, are produced and secreted across the epithelia in enormous quantities by plasma cells in the lamina propria of the intestine (*46–49*). A third component of the barrier is the secretion of a diversity of AMPs (*30*, *31*). AMPs are produced by most epithelial cells but the primary producers are the Paneth cells. These are secretory cells in the small intestine epithelium, located in clusters at the base of crypts. The key role

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

of the Paneth cells in the protection of the epithelia became clear when Paneth cells were specifically ablated in transgenic mice expressing a toxin under the control of a Paneth cell specific promoter. In these mice, both the microbiota and pathogens penetrate the mucosal barrier and the epithelial tissues (*50*). Another study used mutant mice lacking the Nod2 receptor, sensing the bacterial molecule muramyl dipeptide. The Paneth cells in this mutant produce less of the α-defensin AMP (*51*). The Nod2-deficient mice have an increased quantity of symbiotic bacteria in the small intestine (*52*). Thus the AMPs produced by Paneth cells keep the microbiota load under control and contribute to the limitation of the contact between the symbiotic gut bacteria and the epithelial tissues.

Moreover, Paneth cell-produced AMPs also regulate the specific composition of the microbiota in the lumen of the small intestine (*32*). α-defensin production by the Paneth cells can be modulated to produce more or less than the wild type levels by the use of transgenic mice, homozygous or hemizygous for the transgene HD5 encoding a human α-defensin (*53*) and of mice, heterozygous or homozygous for a mutated Mmp-7 allele, required for processing of mouse α-defensin in an active form (*54*). The composition of the microbiota in these complementary models showed reciprocal shifts (*32*). The most notable differences were for bacteria of the Firmicute phylotype which decreased at higher α-defensin production by the Paneth cells while the Bacteroidetes phylotype followed the opposite trend. This is in agreement with an earlier study which found by microscopic observation a shift in the bacterial population in the gut of HD5-expressing mice (*55*). In addition to the shifts in the dominant phylotypes, an important species in the mouse microbiota, known as segmented filamentous bacteria or SFB (formally *Candidatus arthromitis*) is eliminated from the intestinal microbiota in HD5-expressing mice (*32*). Thus Paneth cells and α-defensins control the composition of the intestinal microbiota in a similar way as described above in flies and polyps.

It is well known that the mammalian gut symbionts shape the intestinal and systemic immune system by coordinating the differentiation of pro-inflammatory and anti-inflammatory T cells in the lamina propria (*56*). Specific bacterial species have been identified which stimulate the differentiation of particular T cell types. For example, SFB stimulate the pro-inflammatory T_H17 cells (*8*, *9*) as well as regulatory T cells (*9*). The gut symbiont *Bacteroides fragilis*, through the action of its PSA polysaccharide signal, suppresses T_H17 production and stimulates regulatory T cells (*57*). *Faecalibacterium prausnitzii* (*58*) and a consortium of Clostridium species (*10*) are all stimulating anti-inflammatory regulatory T cells. Thus not surprisingly, the mice models with altered α-defensin production by the Paneth cells and resulting dysbiosis, displayed also an alteration in the T cell production in their lamina propria. Particularly, the notable change in SFB entailed a corresponding change in the T_H17 cell population (*32*). Thus the AMPs also influence, albeit indirectly, the differentiation of the adaptive immune system in the gut.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

Conclusions

The three studies in the very divergent animals Hydra, Drosophila and mouse, using similar strategies, namely the mis-expression of AMP genes in the gut epithelia, come to the same conclusion that the profile and the level of AMPs produced by the gut are crucial to create and maintain a specific and favourable composition of the microbiota. This type of studies has important implications for the understanding of disorders such as inflammatory bowel disease and in particular Crohn's disease. An improper balance between pro- and anti-inflammatory T cells critically affects the onset and the progression of these diseases (*56*). Crohn's disease is also associated with dysbiosis (*59*). Moreover, several risk alleles for Crohn's disease in genetically susceptible people affect the proper functioning of Paneth cells and their ability to secrete AMPs, including mutations in the above mentioned Nod2 gene and other genes affecting the secretory pathway required for AMP secretion (*31*). This correlates with the observed lower production of AMPs in Crohn's disease patients (*55*). Together, these findings provide a coherent picture of Crohn's disease. The presence of risk alleles lead to a reduced Paneth cell functioning and secretion of AMPs which on its turn modifies the composition of the intestinal microbiota and an associated imbalance of pro- and anti-inflammatory responses resulting in or perpetuating the chronic intestinal inflammation associated with the disease (*32*).

AMPs Control Differentiation of Nitrogen Fixing Endosymbionts in Legume Plants

Establishing a Symbiotic Bacterial Population in Legume Nodules

The formation of nodules on the roots of legume plants is activated by the Nod factor signal molecules produced by the rhizobium bacteria in the rhizosphere. Nod factors, recognized by specific membrane-bound receptors in the root epidermal cells, activate proliferation of the root cortical cells and the formation of a so-called nodule primordium. Growth by continuing cell divisions and endoreduplication-driven cell differentiation in the emerging organ leads then ultimately to the formation of a full-grown root nodule (*60*). In parallel with the organogenesis process, Nod factor signalling also initiates the formation of rhizobium-containing infection threads in the root epidermal cells (*13*). The tissues of the growing nodules are invaded by the infection thread network which grows towards the differentiating nodule cells and releases bacteria into their cytoplasm by an endocytotic process (*61*). Single or a few internalized bacteria are confined in organelle-like structures called symbiosomes. The bacterial release, combined with symbiosome multiplication and maturation ultimately fills-up the host cell completely with symbiosomes.

Differentiation of the Symbiotic Rhizobium Bacteria

Within the developing symbiosomes, the rhizobium bacteria differentiate into nitrogen-fixing bacteroids. Differentiated bacteroids are highly specialized

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

bacterial cells, entirely different from bacteria in culture, in the rhizosphere or in infection threads. They are adapted for nitrogen fixation and the existence as an organelle-like entity. This bacterial differentiation is made possible by a massive transcriptome switch and is in part regulated by the FixLJ two-component regulator which senses the low concentration of free-oxygen prevalent in nodules and activates among others the genes required for nitrogen fixation *per se* and for microaerobic respiration (*62*). Moreover, rhizobium differentiation requires a number of bacterial household functions (reviewed in (*63*)) including transport of dicarboxylic acids, amino acids and other nutrients (*17*, *64*, *65*), the lipopolysaccharide component of the bacterial envelop (*66*, *67*) and a performant oxidative stress resistance mechanism (*68*).

In many but not all legume plants, bacteroid formation is also associated with drastic modifications of the bacterial morphology and cytosol organization. In plants of the Inverted Repeat Lacking Clade (IRLC) which includes the model legume *Medicago truncatula*, bacteroids are considerably elongated, reaching a length up to 10 µm, and can sometimes be branched. These bacteroids have altered membrane permeability and a high amplification of their genome which is condensed in multiple nucleoids of variable size (*23*, *69*). The intensive DNA amplification in bacteroids requires an unusual oxidative-stress-resistant, cobalamine-dependent ribonucleotide reductase for the synthesis of the required deoxy nucleotides (*70*). The polyploidy of the genome suggests that the bacteroid differentiation process includes an interference with the bacterial cell cycle. This process of bacteroid differentiation is irreversible (or terminal) since these differentiated bacteria cannot produce offspring. On the other hand, in other legume groups the morphology, membrane integrity and genome content of bacteroids are similar to those of free-living bacteria. These bacteroids can produce offspring and are therefore reversibly differentiated (*23*). While terminal differentiation of bacteroids is thus not essential *per se* for symbiotic nitrogen fixation, it possibly improves the symbiotic efficiency of the bacteroids as suggested by a comparison of the symbiotic performance of terminal and reversible bacteroids (*71*). However, this single case study should be extended with a more extensive comparison between both bacteroid types before a general conclusion can be made.

The AMP-like NCR Peptide Family

The terminal differentiation of *Sinorhizobium meliloti* in *M. truncatula* nodules is independent of the FixLJ-controlled physiological bacteroid differentiation pathway because bacterial mutants in this signalling pathway still display features of terminal bacteroid differentiation (*72*). On the other hand, it was found by using nearly isogenic rhizobium strains nodulating both IRLC and non-IRLC legume species, that the host rather than the bacterial genetic repertoire determines the terminal bacterial differentiation. Therefore, it was concluded that the host cells of IRLC legume nodules produce factors that direct the terminal differentiation of rhizobium in the symbiosomes (*23*). A transcriptome search in *M. truncatula* designed to identify those factors was based on the assumptions that the encoding genes were induced during the nodule organogenesis and expressed in the infected nodule cells. Moreover, homologous genes were expected to be similarly expressed in the nodules of other IRLC legumes but not in plants with reversible bacteroid differentiation. Nodule-specific cysteine-rich (NCR) peptides were identified by this approach as likely candidates.

Remarkably, the NCR gene family in *M. truncatula* encodes more than 450 different peptides which are most similar to defensin-like AMPs. Homologs have been found in other IRLC legumes but not in species forming nodules with reversibly differentiated bacteroids (*73*, *74*). Equally remarkable, the expression of all the *M. truncatula* NCR genes, except for two which are also expressed in roots, is strictly nodule-specific (*73*, *74*). Expression was not found in any other plant organ or in other biotic interactions with mycorrhizal fungi, rhizosphere bacteria, pathogens, nematodes or insects. Transcriptome analysis of nodules obtained with a large collection of symbiotic mutants of *M. truncatula* and of its bacterial partner *S. meliloti* revealed that NCR gene expression was correlated with symbiotic cell formation (*72*). Moreover, in the case of examples tested with *in situ* hybridization or promoter-GUS fusion, the expression of NCR genes was found to be restricted to the rhizobium-infected plant cells. However, different subsets of NCR genes had distinct expression domains, certain being expressed in young symbiotic cells while others in older or mature symbiotic cells (*74*). Some NCR genes are expressed at very high levels and based on EST counts, it was estimated that the combined transcripts of the NCR family constitute close to 5% of the total mRNA pool in nodules.

Although the NCRs have only limited sequence homology with other peptides, their protein structure, gene organization and family structure resembles AMPs. The possible involvement of AMP-like peptides in bacteroid differentiation is meaningful in light of certain bacteroid features such as membrane modifications, inhibition of cytokinesis and inability to reproduce which are known effects of different types of AMPs. In the NCR peptide sequences, an N-terminal signal peptide can be recognized, which is a cleavable tag for targeting of the peptide in the secretory pathway. By expressing protein fusions of NCRs or their signal peptides in a heterologous system, it was shown that these signal peptides are indeed functional and mediate entry of the peptides in the secretory pathway (*74*). The mature NCR peptides (the C-terminal part of the proteins obtained after the cleavage of the signal peptide during translocation in the endoplasmic reticulum) are around 40 amino acids long and are typified by a conserved pattern of cysteine residues while the rest of the sequence is highly variable amongst the family members in contrast to the preserved signal peptides (*74*). This reflects an evolutionary pattern with the signal peptides subjected to purifying selection in contrast to the mature peptides which were subjected to diversifying selection (*73*). Similar observations are frequently made for AMP gene families, including conservation of signal peptides and cysteine residues but strong divergence in the functional mature peptide (*75*, *76*).

Interaction of NCR Peptides with Endosymbiotic Rhizobium

Peptides in the size range expected for NCRs accumulate in the nodules and co-purify with the bacteroids. Specific antibodies and peptide sequencing

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

confirmed that these peptides correspond to NCRs. Moreover, *in situ* localization of the NCR peptides confirmed this localization and demonstrated that at least some NCRs are transported in the bacteroid cytosol (*77*). Thus NCRs are targeted to and accumulate in high amounts in symbiosomes and bacteroids and this localization of the peptides indicates that the bacteroids are their targets. Since symbiosomes are vesicles with a plant-derived membrane, protein transport towards the symbiosomes and bacteroids depends on the secretory pathway (*72*, *77–79*). Thus the localization of the NCRs and the presence of the characteristic signal peptide are in agreement with a transport mechanism of the peptides through the secretory pathway. This was confirmed by the analysis of the *M. truncatula dnf1* mutant. This mutant is deficient in a nodule-specific subunit of the signal peptidase complex of the secretory pathway. This endoplasmic reticulum-located enzyme complex is responsible for the proteolitic cleavage of the signal peptide of secretory proteins which is critical for their correct targeting (*80*). The *dnf1* mutant forms non-functional nodules which contain infected nodule cells. However, the symbiosome bacteria do not differentiate into elongated bacteroids (*77*, *79*). In this mutant, the signal peptide of the NCRs is not properly processed and by consequence the peptides are blocked in the endoplasmic reticulum and are not targeted to the bacteroids. Thus, obstructing NCR transport is correlated with the absence of bacteroid differentiation in the symbiosomes, in agreement with a role of the NCRs in this bacterial differentiation process (*77*). The transcriptome analysis of nodules and infected cells in *M. truncatula* has revealed that the majority of the up-regulated genes in these cells are involved in protein secretion (*72*). These cells have a remarkably well developed and abundant endoplasmic reticulum (*72*). The genes encoding the four conserved subunits of the signal peptidase complex (including *Dnf1*) are duplicated in the *M. truncatula* genome and one copy maintains a ubiquitous expression while the other one acquired a nodule-specific expression (*79*). Thus the symbiosome-containing nodule cells are highly specialized for protein, and particularly for NCR transport to the symbiosomes (*72*, *79*, *81*).

In an opposite, gain-of-function approach, NCR genes were ectopically expressed in the infected nodule cells of *Lotus japonicus*, a legume with reversible bacteroid differentiation and lacking NCR genes. Expression of certain *NCR* genes was sufficient to induce features of terminal bacteroid differentiation with symbiosomes containing single and remarkably elongated bacteroids (*77*). Even *in vitro* application of NCR peptides to *S. meliloti* free-living bacteria results in bacteroid-like features, notably in high permeability of the membrane, inhibition of bacterial proliferation, DNA accumulation and cell elongation (*77*, *82*).

All together, these findings are in agreement with a major role of the NCR peptides in the terminal differentiation of the symbiosome-located rhizobia.

Bacterial Protection against the Antimicrobial Activity of NCRs

NCRs are similar to AMPs such as defensins and the analysis of the *in vitro* NCR activity demonstrated that some NCR peptides indeed possess genuine antimicrobial properties and effectively kill not only *S. meliloti* (*77*) but also both gram-positive and gram-negative bacteria (E. Kondorosi, unpublished

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

data) at similar concentrations as most other antimicrobial peptides. The *in vivo* and *in vitro* effects of NCRs on *S. meliloti* are dramatically different because NCR-challenged bacteroids in nodule symbiosomes maintain an active metabolism for nitrogen fixation, despite their inhibition for growth. This could be explained by a concerted *in vivo* action of several tens or hundreds of peptides, each likely present at very low concentration. This is hardly comparable to the *in vitro* effect of the peptides externally applied at high concentrations. Furthermore, particular conditions prevalent in nodules such as the low free oxygen concentration which is needed for the activity of the oxygen-sensitive nitrogenase enzyme could modulate the bacterial responses to the NCRs in such a way that the bacteroids remain alive, although with a complete loss of their reproductive capacity.

Moreover, the *S. meliloti* BacA protein was identified as a factor that helps the symbiosome bacteria to survive the NCR exposure (*82*). The *S. meliloti bacA* gene was originally identified as an essential gene for the establishment of an effective symbiosis (*83*). *BacA* mutants induce nodule formation on *Medicago* plants and the bacteria are released from infection threads into the symbiotic nodule cells but they fail to differentiate into elongated, nitrogen-fixing bacteroids (*72*, *83*). The BacA protein is conserved in many bacteria, including the rhizobia and interestingly, the protein is critical for effective symbiosis and bacteroid development in those legume hosts that produce NCR peptides such as *Pisum sativum* (pea) and *Astragalus sinicus* (IRLC legumes) but it is dispensable for this process in *Phaseolus vulgaris* (bean), *Vigna unguiculata* (cowpea), and *L. japonicus*, which are legume hosts naturally devoid in these peptides (*84–89*). The BacA protein is an integral membrane protein in bacteria that belongs to the ATP binding cassette (ABC) superfamily of membrane transporters. The transported substrate remains unknown although the BacA protein and its *Escherichia coli* homolog SbmA facilitate the uptake of proline-rich peptides suggesting that those proteins can function as peptide transporters (*90*, *91*). *S. meliloti* BacA also affects the incorporation of unusual, very long chain fatty acids in the lipo-polysaccharide membrane (*92*) and *Rhizobium leguminosarum* bv. *viciae* BacA affects the transcription of membrane proteins. Thus BacA has pleiotropic effects on the bacterial envelop.

The BacA-deficient mutant of *S. meliloti in vitro* was still able to induce cell enlargement and DNA amplification in response to NCR peptides at low concentrations suggesting that the protein is not essential for the differentiation process *per se* despite the inability of the *bacA* mutant to differentiate *in planta*. However, the mutant was hypersensitive for the antimicrobial activity of the NCR peptides applied at higher concentrations indicating that the BacA protein provides resistance to *S. meliloti* against the antimicrobial activity of the NCRs (*82*). Within the symbiosomes of *M. truncatula* nodules, the *bacA* mutant bacteria are similarly challenged with NCRs as the wild type bacteria but the mutant, contrary to the wild type strain, cannot survive the symbiosome environment. However, in the *dnf1* mutant of *M. truncatula* where NCR transport to the symbiosomes is blocked, BacA is not critical anymore for bacterial survival in the symbiosomes. Thus BacA is required in symbiosis by protecting *S. meliloti* against the bactericidal effects of NCRs in *M. truncatula* nodules and thereby

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

enabling proper bacteroid development (*82*). The mechanism for this protection remains to be discovered. It could be related to a peptide transport function of the BacA protein or alternatively, it could be related to bacterial envelop features affected by BacA which are critical for the amount of membrane damage induced by the NCR peptides.

Interestingly, BacA function is also crucial for the pathogenicity of bacteria such as *Brucella* and *Mycobacterium* (*85*, *93*). These pathogens establish chronic infections in animal hosts where they need to withstand cocktails of AMPs to survive. The *E. coli* and *Brucella bacA* genes can complement the symbiotic defect of the *S. meliloti bacA* mutant (*94*, *95*) and the *Brucella* gene can protect the mutant against the antimicrobial activity of NCRs (*82*). Thus BacA function is conserved and BacA-mediated protection of bacteria against host AMPs may be general and a critical stage in the establishment of symbiotic as well as pathogenic chronic host infections.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

Future Directions: What Is the Biological Meaning of Bacterial Differentiation?

The different lines of evidence described above demonstrate that the NCR peptides are the principal mediators of bacteroid differentiation in *M. truncatula* symbiosomes. However, many unsolved questions are remaining, notably with respect to the mode of action of the peptides and to the biological meaning of the NCR-induced effects. Some NCR peptides inhibit *in vivo* and *in vitro* bacterial division leading to cell elongation. Such NCRs were localized at the division site of *S. meliloti* cells (*77*) suggesting that these peptides may interfere with the bacterial cell division machinery. However, the high sequence variety of NCRs suggests diversity in their functions, mode of actions and bacterial targets. Perhaps some peptides interfere with bacteroid metabolism and thereby optimize the efficiency of the nitrogen fixation as suggested by the higher symbiotic efficiency of terminal bacteroids as compared to reversible bacteroids (*71*). For example, the accumulation of the storage compound polyhydroxybutirate (PHB), which takes the host-supplied carbon away from nitrogen fixation, is very frequently observed in bacteroids but not in the bacteroids of IRLC legumes. Inhibition of PHB accumulation in those bacteroids could be a direct or indirect consequence of the terminal differentiation. Another raison d'être for the high diversity of NCR peptides could be an adaptation to the high diversity of rhizobia in soils. The diversifying selection that has shaped the NCR family is compatible with such a hypothesis (*73*).

Additional, non-exclusive hypotheses can be put forward as to explain a better performance of terminally differentiated bacteroids. Polyploidy of the bacteroids may support higher metabolic activity of the cells in a general way as it is in the case of eukaryotic cells (*60*). Terminally differentiated bacteroids are always present as a single bacterium per symbiosome which may permit a very efficient nutrient exchange with the host cell. On the contrary, multiple reversibly differentiated bacteroids are present in a single symbiosome and these bacteroids have therefore a more limited and less efficient contact with the symbiosome membrane. Moreover, terminally differentiated bacteroids are effectively digested by the host during the senescence process, at the end of the nodule life. This is less so for nodules containing reversibly differentiated bacteroids which can efficiently survive nodule senescence. Thus, nutrient recycling during senescence might provide an advantage to the plant.

Concluding Remarks

The illustrated examples in Hydra, Drosophila and mouse strongly suggest that AMPs controlling gut microbiota is most likely conserved in all branches of the animal tree of life. AMPs also control endosymbiotic bacteria as illustrated by the legume bacteroids. It would thus not come as a surprise to find the implication of AMPs also in other symbiotic systems as for example in the formation of the light organ in the *V. fischeri*-squid symbiosis or in the insect bacteriomes such as the one formed during the aphid symbiosis with *Buchnera*. A specific transcriptome analysis of the bacteriome in the weevil *Sitophilus zeamais* indeed identified the specific transcription of an AMP gene in bacteriomes (*96*). Terminally differentiated bacteroids are not only observed in IRLC legumes but in other legume clades as well (*97*). For example, spherical bacteroids are described in *Aeschynomene* species (*98*). Also here, bacteroid differentiation is induced by plant factors whose nature however remains to be discovered.

Analyzing more symbiotic systems and the interaction of host AMPs and more generally the innate and adaptive immune systems with the microbial symbionts may change our thinking on the evolutionary roots of these immune systems. Did they evolve to respond to pathogens or rather to select and maintain symbiotic partners?

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

References

1. Turnbaugh, P. J.; Ley, R. E.; Hamady, M.; Fraser-Liggett, C. M.; Knight, R.; Gordon, J. I. *Nature* **2007**, *449*, 804–810.
2. Buchon, N.; Broderick, N. A.; Chakrabarti, S.; Lemaitre, B. *Genes Dev.* **2009**, *23*, 2333–2344.
3. Rawls, J. F.; Samuel, B. S.; Gordon, J. I. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4596–4601.
4. Bates, J. M.; Mittge, E.; Kuhlman, J.; Baden, K. N.; Cheesman, S. E.; Guillemin, K. *Dev. Biol.* **2006**, *297*, 374–386.
5. Hooper, L. V.; Wong, M. H.; Thelin, A.; Hansson, L.; Falk, P. C.; Gordon, J. I. *Science* **2001**, *291*, 881–884.
6. Stappenbeck, T. S.; Hooper, L. V.; Gordon, J. I. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15451–15455.
7. Mazmanian, S. K.; Liu, C. H.; Tzianabos, A. O.; Kasper, D. L. *Cell* **2005**, *122*, 107–118.
8. Ivanov, I. I.; Atarashi, K.; Manel, N.; Brodie, E. L.; Shima, T.; Karaoz, U.; Wei, D. G.; Goldfarb, K. C.; Santee, C. A.; Lynch, S. V.; Tanoue, T.; Imaoka, A.; Itoh, K.; Takeda, K.; Umesaki, Y.; Honda, K.; Littman, D. R. *Cell* **2009**, *139*, 485–498.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

9. Gaboriau-Routhiau, V.; Rakotobe, S.; Lecuyer, E.; Mulder, I.; Lan, A.; Bridonneau, C.; Rochet, V.; Pisi, A.; De Paepe, M.; Brandi, G.; Eberl, G.; Snel, J.; Kelly, D.; Cerf-Bensussan, N. *Immunity* **2009**, *31*, 677–689.
10. Atarashi, K.; Tanoue, T.; Shima, T.; Imaoka, A.; Kuwahara, T.; Momose, Y.; Cheng, G. H.; Yamasaki, S.; Saito, T.; Ohba, Y.; Taniguchi, T.; Takeda, K.; Hori, S.; Ivanov, I. I.; Umesaki, Y.; Itoh, K.; Honda, K. *Science* **2011**, *331*, 337–341.
11. Nyholm, S. V.; McFall-Ngai, M. J. *Nat. Rev. Microbiol.* **2004**, *2*, 632–642.
12. Braendle, C.; Miura, T.; Bickel, R.; Shingleton, A. W.; Kambhampati, S.; Stern, D. L. *PLoS Biol.* **2003**, *1*, 70–76.
13. Oldroyd, G. E. D.; Downie, J. M. *Annu. Rev. Plant Biol.* **2008**, *59*, 519–546.
14. Backhed, F.; Ley, R. E.; Sonnenburg, J. L.; Peterson, D. A.; Gordon, J. I. *Science* **2005**, *307*, 1915–1920.
15. Warnecke, F.; Luginbuhl, P.; Ivanova, N.; Ghassemian, M.; Richardson, T. H.; Stege, J. T.; Cayouette, M.; McHardy, A. C.; Djordjevic, G.; Aboushadi, N.; Sorek, R.; Tringe, S. G.; Podar, M.; Martin, H. G.; Kunin, V.; Dalevi, D.; Madejska, J.; Kirton, E.; Platt, D.; Szeto, E.; Salamov, A.; Barry, K.; Mikhailova, N.; Kyrpides, N. C.; Matson, E. G.; Ottesen, E. A.; Zhang, X. N.; Hernandez, M.; Murillo, C.; Acosta, L. G.; Rigoutsos, I.; Tamayo, G.; Green, B. D.; Chang, C.; Rubin, E. M.; Mathur, E. J.; Robertson, D. E.; Hugenholtz, P.; Leadbetter, J. R. *Nature* **2007**, *450*, 560–565.
16. Shigenobu, S. S. S.; Wilson, A. C. C. *Cell. Mol. Life Sci.* **2011**, *68*, 1297–1309.
17. White, J.; Prell, J.; James, E. K.; Poole, P. *Plant Physiol.* **2007**, *144*, 604–614.
18. Tringe, S. G.; von Mering, C.; Kobayashi, A.; Salamov, A. A.; Chen, K.; Chang, H. W.; Podar, M.; Short, J. M.; Mathur, E. J.; Detter, J. C.; Bork, P.; Hugenholtz, P.; Rubin, E. M. *Science* **2005**, *308*, 554–557.
19. Koropatnick, T. A.; Engle, J. T.; Apicella, M. A.; Stabb, E. V.; Goldman, W. E.; McFall-Ngai, M. J. *Science* **2004**, *306*, 1186–1188.
20. Yip, E. S.; Grublesky, B. T.; Hussa, E. A.; Visick, K. L. *Mol. Microbiol.* **2005**, *57*, 1485–1498.
21. Mandel, M. J.; Wollenberg, M. S.; Stabb, E. V.; Visick, K. L.; Ruby, E. G. *Nature* **2009**, *458*, 215–218.
22. Wier, A. M.; Nyholm, S. V.; Mandel, M. J.; Massengo-Tiasse, R. P.; Schaefer, A. L.; Koroleva, I.; Splinter-BonDurant, S.; Brown, B.; Manzella, L.; Snir, E.; Almabrazi, H.; Scheetz, T. E.; Bonaldo, M. D.; Casavant, T. L.; Soares, M. B.; Cronan, J. E.; Reed, J. L.; Ruby, E. G.; McFall-Ngai, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 2259–2264.
23. Mergaert, P.; Uchiumi, T.; Alunni, B.; Evanno, G.; Cheron, A.; Catrice, O.; Mausset, A. E.; Barloy-Hubler, F.; Galibert, F.; Kondorosi, A.; Kondorosi, E. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 5230–5235.
24. Ley, R. E.; Peterson, D. A.; Gordon, J. I. *Cell* **2006**, *124*, 837–848.
25. Ley, R. E.; Hamady, M.; Lozupone, C.; Turnbaugh, P. J.; Ramey, R. R.; Bircher, J. S.; Schlegel, M. L.; Tucker, T. A.; Schrenzel, M. D.; Knight, R.; Gordon, J. I. *Science* **2008**, *320*, 1647–1651.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

26. Ochman, H.; Worobey, M.; Kuo, C. H.; Ndjango, J. B. N.; Peeters, M.; Hahn, B. H.; Hugenholtz, P. *PLoS Biol.* **2010**, *8*, e1000546.
27. Rawls, J. F.; Mahowald, M. A.; Ley, R. E.; Gordon, J. I. *Cell* **2006**, *127*, 423–433.
28. Ryu, J. H.; Kim, S. H.; Lee, H. Y.; Bai, J. Y.; Nam, Y. D.; Bae, J. W.; Lee, D. G.; Shin, S. C.; Ha, E. M.; Lee, W. J. *Science* **2008**, *319*, 777–782.
29. Fraune, S.; Bosch, T. C. G. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 13146–13151.
30. Hooper, L. V.; Macpherson, A. J. *Nat. Rev. Immunol.* **2010**, *10*, 159–169.
31. Bevins, C. L.; Salzman, N. H. *Nat. Rev. Microbiol.* **2011**, *9*, 356–368.
32. Salzman, N. H.; Hung, K. C.; Haribhai, D.; Chu, H. T.; Karlsson-Sjoberg, J.; Amir, E.; Teggatz, P.; Barman, M.; Hayward, M.; Eastwood, D.; Stoel, M.; Zhou, Y. J.; Sodergren, E.; Weinstock, G. M.; Bevins, C. L.; Williams, C. B.; Bos, N. A. *Nat. Immunol.* **2010**, *11*, 76–83.
33. Fraune, S.; Augustin, R.; Anton-Erxleben, F.; Wittlieb, J.; Gelhaus, C.; Klimovich, V. B.; Samoilovich, M. P.; Bosch, T. C. G. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 18067–18072.
34. Maróti, G.; Kereszt, A.; Kondorosi, E.; Mergaert, P. *Res. Microbiol.* **2011**, *162*, 363–374.
35. Hancock, R. E.; Sahl, H. G. *Nat. Biotechnol* **2006**, *24*, 1551–7.
36. Fraune, S.; Abe, Y.; Bosch, T. C. G. *Environ. Microbiol.* **2009**, *11*, 2361–2369.
37. Augustin, R.; Anton-Erxleben, F.; Jungnickel, S.; Hemmrich, G.; Spudy, B.; Podschun, R.; Bosch, T. C. G. *Antimicrob. Agents Chemother.* **2009**, *53*, 5245–5250.
38. Augustin, R.; Siebert, S.; Bosch, T. C. G. *Dev. Comp. Immunol.* **2009**, *33*, 830–837.
39. Bosch, T. C. G.; Augustin, R.; Anton-Erxleben, F.; Fraune, S.; Hemmrich, G.; Zill, H.; Rosenstiel, P.; Jacobs, G.; Schreiber, S.; Leippe, M.; Stanisak, M.; Grotzinger, J.; Jung, S.; Podschun, R.; Bartels, J.; Harder, J.; Schroder, J. M. *Dev. Comp. Immunol.* **2009**, *33*, 559–569.
40. Jung, S.; Dingley, A. J.; Augustin, R.; Anton-Erxleben, F.; Stanisak, M.; Gelhaus, C.; Gutsmann, T.; Hammer, M. U.; Podschun, R.; Bonvin, A.; Leippe, M.; Bosch, T. C. G.; Grotzinger, J. *J. Biol. Chem.* **2009**, *284*, 1896–1905.
41. Lemaitre, B.; Hoffmann, J. *Annu. Rev. Immunol.* **2007**, *25*, 697–743.
42. Lengyel, J. A.; Iwaki, D. D. *Dev. Biol.* **2002**, *243*, 1–19.
43. Ryu, J. H.; Nam, K. B.; Oh, C. T.; Nam, H. J.; Kim, S. H.; Yoon, J. H.; Seong, J. K.; Yoo, M. A.; Jang, I. H.; Brey, P. T.; Lee, W. J. *Mol. Cell. Biol.* **2004**, *24*, 172–185.
44. Johansson, M. E. V.; Larsson, J. M. H.; Hansson, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 4659–4665.
45. Johansson, M. E. V.; Phillipson, M.; Petersson, J.; Velcich, A.; Holm, L.; Hansson, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 15064–15069.
46. Hapfelmeier, S.; Lawson, M. A. E.; Slack, E.; Kirundi, J. K.; Stoel, M.; Heikenwalder, M.; Cahenzli, J.; Velykoredko, Y.; Balmer, M. L.; Endt, K.;

Geuking, M. B.; Curtiss, R.; McCoy, K. D.; Macpherson, A. J. *Science* **2010**, *328*, 1705–1709.

47. Macpherson, A. J.; Gatto, D.; Sainsbury, E.; Harriman, G. R.; Hengartner, H.; Zinkernagel, R. M. *Science* **2000**, *288*, 2222–2226.
48. Peterson, D. A.; McNulty, N. P.; Guruge, J. L.; Gordon, J. I. *Cell Host Microbe* **2007**, *2*, 328–339.
49. Suzuki, K.; Meek, B.; Doi, Y.; Muramatsu, M.; Chiba, T.; Honjo, T.; Fagarasan, S. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 1981–1986.
50. Vaishnava, S.; Behrendt, C. L.; Ismail, A. S.; Eckmann, L.; Hooper, L. V. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 20858–20863.
51. Kobayashi, K. S.; Chamaillard, M.; Ogura, Y.; Henegariu, O.; Inohara, N.; Nunez, G.; Flavell, R. A. *Science* **2005**, *307*, 731–734.
52. Petnicki-Ocwieja, T.; Hrncir, T.; Liu, Y. J.; Biswas, A.; Hudcovic, T.; Tlaskalova-Hogenova, H.; Kobayashi, K. S. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 15813–15818.
53. Salzman, N. H.; Ghosh, D.; Huttner, K. M.; Paterson, Y.; Bevins, C. L. *Nature* **2003**, *422*, 522–526.
54. Wilson, C. L.; Ouellette, A. J.; Satchell, D. P.; Ayabe, T.; Lopez-Boado, Y. S.; Stratman, J. L.; Hultgren, S. J.; Matrisian, L. M.; Parks, W. C. *Science* **1999**, *286*, 113–117.
55. Wehkamp, J.; Salzman, N. H.; Porter, E.; Nuding, S.; Weichenthal, M.; Petras, R. E.; Shen, B.; Schaeffeler, E.; Schwab, M.; Linzmeier, R.; Feathers, R. W.; Chu, H. T.; Lima, H.; Fellermann, K.; Ganz, T.; Stange, E. F.; Bevins, C. L. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 18129–18134.
56. Round, J. L.; Mazmanian, S. K. *Nat. Rev. Immunol.* **2009**, *9*, 313–323.
57. Mazmanian, S. K.; Round, J. L.; Kasper, D. L. *Nature* **2008**, *453*, 620–625.
58. Sokol, H.; Pigneur, B.; Watterlot, L.; Lakhdari, O.; Bermudez-Humaran, L. G.; Gratadoux, J. J.; Blugeon, S.; Bridonneau, C.; Furet, J. P.; Corthier, G.; Grangette, C.; Vasquez, N.; Pochart, P.; Trugnan, G.; Thomas, G.; Blottiere, H. M.; Dore, J.; Marteau, P.; Seksik, P.; Langella, P. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 16731–16736.
59. Frank, D. N.; Amand, A. L. S.; Feldman, R. A.; Boedeker, E. C.; Harpaz, N.; Pace, N. R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 13780–13785.
60. Kondorosi, E.; Kondorosi, A. *FEBS Lett* **2004**, *567*, 152–157.
61. Brewin, N. J. *Crit. Rev. Plant Sciences* **2004**, *23*, 293–316.
62. Bobik, C.; Meilhoc, E.; Batut, J. *J. Bacteriol.* **2006**, *188*, 4890–4902.
63. Kereszt, A.; Mergaert, P.; Kondorosi, E. *Mol. Plant-Microbe Interact.* **2011**, doi:10.1094/MPMI-06-11-0152.
64. Prell, J.; White, J. P.; Bourdes, A.; Bunnewell, S.; Bongaerts, R. J.; Poole, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 12477–12482.
65. Mulley, G.; White, J. P.; Karunakaran, R.; Prell, J.; Bourdes, A.; Bunnewell, S.; Hill, L.; Poole, P. S. *Mol. Microbiol.* **2011**, *80*, 149–167.
66. Haag, A. F.; Wehmeier, S.; Beck, S.; Marlow, V. L.; Fletcher, V.; James, E. K.; Ferguson, G. P. *J. Bacteriol.* **2009**, *191*, 4681–4686.
67. Campbell, G. R. O.; Reuhs, B. L.; Walker, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 3938–3943.
68. Chang, C.; Damiani, I.; Puppo, A.; Frendo, P. *Mol. Plant* **2009**, *2*, 370–377.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

69. Vasse, J.; de Billy, F.; Camut, S.; Truchet, G. *J. Bacteriol.* **1990**, *172*, 4295–306.
70. Taga, M. E.; Walker, G. C. *Mol. Plant-Microbe Interact.* **2010**, *23*, 1643–1654.
71. Oono, R.; Denison, R. F. *Plant Physiol* **2010**, *154*, 1541–1548.
72. Maunoury, N.; Redondo-Nieto, M.; Bourcy, M.; Van de Velde, W.; Alunni, B.; Laporte, P.; Durand, P.; Agier, N.; Marisa, L.; Vaubert, D.; Delacroix, H.; Duc, G.; Ratet, P.; Aggerbeck, L.; Kondorosi, E.; Mergaert, P. *PLoS ONE* **2010**, *5*, e9519.
73. Alunni, B.; Kevei, Z.; Redondo-Nieto, M.; Kondorosi, A.; Mergaert, P.; Kondorosi, E. *Mol. Plant-Microbe Interact.* **2007**, *20*, 1138–1148.
74. Mergaert, P.; Nikovics, K.; Kelemen, Z.; Maunoury, N.; Vaubert, D.; Kondorosi, A.; Kondorosi, E. *Plant Physiol.* **2003**, *132*, 161–173.
75. Patil, A.; Hughes, A. L.; Zhang, G. L. *Physiol. Genomics* **2004**, *20*, 1–11.
76. Semple, C. A.; Gautier, P.; Taylor, K.; Dorin, J. R. *Mol. Diversity.* **2006**, *10*, 575–584.
77. Van de Velde, W.; Zehirov, G.; Szatmari, A.; Debreczeny, M.; Ishihara, H.; Kevei, Z.; Farkas, A.; Mikulass, K.; Nagy, A.; Tiricz, H.; Satiat-Jeunemaitre, B.; Alunni, B.; Bourge, M.; Kucho, K.; Abe, M.; Kereszt, A.; Maroti, G.; Uchiumi, T.; Kondorosi, E.; Mergaert, P. *Science* **2010**, *327*, 1122–1126.
78. Limpens, E.; Ivanov, S.; van Esse, W.; Voets, G.; Fedorova, E.; Bisseling, T. *Plant Cell* **2009**, *21*, 2811–2828.
79. Wang, D.; Griffitts, J.; Starker, C.; Fedorova, E.; Limpens, E.; Ivanov, S.; Bisseling, T.; Long, S. *Science* **2010**, *327*, 1126–1129.
80. Paetzel, M.; Karla, A.; Strynadka, N. C.; Dalbey, R. E. *Chem. Rev.* **2002**, *102*, 4549–80.
81. Wang, D.; Dong, X. *Mol. Plant* **2011**, *4*, 581–587.
82. Haag, A. F.; Baloban, M.; Sani, M.; Kerscher, B.; Pierre, O.; Farkas, A.; Longhi, R.; Boncompagni, E.; Hérouart, D.; Dall'Angelo, S.; Kondorosi, E.; Zanda, M.; Mergaert, P.; Ferguson, G. P. *PLoS Biol.* **2011**, *9*, e1001169.
83. Glazebrook, J.; Ichige, A.; Walker, G. C. *Genes Dev.* **1993**, *7*, 1485–97.
84. Ardissone, S.; Kobayashi, H.; Kambara, K.; Rummel, C.; Noel, K. D.; Walker, G. C.; Broughton, W. J.; Deakin, W. J. *J. Bacteriol.* **2011**, *193*, 2218–2228.
85. Domenech, P.; Kobayashi, H.; LeVier, K.; Walker, G. C.; Barry, C. E. *J. Bacteriol.* **2009**, *191*, 477–485.
86. Karunakaran, R.; Haag, A. F.; East, A. K.; Ramachandran, V. K.; Prell, J.; James, E. K.; Scocchi, M.; Ferguson, G. P.; Poole, P. S. *J. Bacteriol.* **2010**, *192*, 2920–2928.
87. LeVier, K.; Walker, G. C. *J. Bacteriol.* **2001**, *183*, 6444–6453.
88. Maruya, J.; Saeki, K. *Plant Cell Physiol.* **2010**, *51*, 1443–1452.
89. Tan, X. J.; Cheng, Y.; Li, Y. X.; Li, Y. G.; Zhou, J. C. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 519–526.
90. Marlow, V. L.; Haag, A. F.; Kobayashi, H.; Fletcher, V.; Scocchi, M.; Walker, G. C.; Ferguson, G. P. *J. Bacteriol.* **2009**, *191*, 1519–1527.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

91. Mattiuzzo, M.; Bandiera, A.; Gennaro, R.; Benincasa, M.; Pacor, S.; Antcheva, N.; Scocchi, M. *Mol. Microbiol.* **2007**, *66*, 151–163.
92. Ferguson, G. P.; Datta, A.; Baumgartner, J.; Roop, R. M., 2nd; Carlson, R. W.; Walker, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 5012–5017.
93. LeVier, K.; Phillips, R. W.; Grippe, V. K.; Roop, R. M.; Walker, G. C. *Science* **2000**, *287*, 2492–2493.
94. Ichige, A.; Walker, G. C. *J. Bacteriol.* **1997**, *179*, 209–216.
95. Wehmeier, S.; Arnold, M. F. F.; Marlow, V. L.; Aouida, M.; Myka, K. K.; Fletcher, V.; Benincasa, M.; Scocchi, M.; Ramotar, D.; Ferguson, G. P. *Microbiology* **2010**, *156*, 2702–2713.
96. Anselme, C.; Perez-Brocal, V.; Vallier, A.; Vincent-Monegat, C.; Charif, D.; Latorre, A.; Moya, A.; Heddi, A. *BMC Biol.* **2008**, *6*, 43.
97. Oono, R.; Schmitt, I.; Sprent, J. I.; Denison, R. F. *New Phytol.* **2010**, *187*, 508–520.
98. Bonaldi, K.; Gargani, D.; Prin, Y.; Fardoux, J.; Gully, D.; Nouwen, N.; Goormachtig, S.; Giraud, E. *Mol. Plant-Microbe Interact.* **2011**, doi:10.1094/MPMI-04-11-0093.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch011

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Chapter 12

Antimicrobial Peptides for Plant Disease Control. From Discovery to Application

Emilio Montesinos,*[,1] Esther Badosa,[1] Jordi Cabrefiga,[1] Marta Planas,[2] Lidia Feliu,[2] and Eduard Bardají[2]

[1]Laboratory of Plant Pathology, Institute of Food and Agricultural Technology-CIDSAV-XaRTA, University of Girona, 17071 Girona, Spain
[2]LIPPSO, Department of Chemistry; University of Girona, Campus Montilivi, 17071 Girona, Spain
***E-mail: emilio.montesinos@udg.edu; emonte@intea.udg.edu**

Sustainable protection of plant crops against diseases relies on a rational use of pesticides and to a reduction of the number of active ingredients to the more selective, less toxic and with a lower negative environmental impact. Antimicrobial peptides (AMPs) are envisaged as new plant disease control products because of the need for novel antifungals, antibacterials and plant strengtheners in Agriculture. Natural AMPs are produced in low amounts, some are toxic, or low active or unstable, and require generally complex and costly procedures for extraction and purification from the producing organism. Synthetic AMPs offer alternatives but require pharma approaches for development as plant protection products. We have developed linear undecapeptides (CECMEL11) and cyclic decapeptides (CYC10) against plant pathogens following the classical hit-to-lead and lead optimization approaches, based on combinatorial chemistry of some critical positions in their amino acid sequence. The peptides were improved for activity against plant pathogenic bacteria and fungi, but minimizing hemolytic activity and protease susceptibility. The selected peptides covered a wide range of action spectrum. Peptide BP100 was strongly lytic against Gram negative bacteria including plant pathogenic bacteria and food-borne human bacterial pathogens, but poorly antifungal. Peptide BP21 displayed strong fungicidal and sporicidal activity but slight antibacterial activity. Acute

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

oral toxicity in mice is slight or non-toxic. Proof-of-concept assays have been performed with success including whole plant tests against *Erwinia amylovora* and *Pseudomonas syringae* on pear, and *Xanthomonas axonopodis* pv. *vesicatoria* on pepper, as well as in postharvest against *Penicillium*-rot on apple. The main limitation of the implementation of the antimicrobial peptide technology in the field of plant protection is due to the production costs, but strong efforts are in progress to produce these peptides using microbial and plant biofactories.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

1. Introduction

Losses in crop production due to plant disease caused by 120 genera of fungi, 30 types of viruses, and 10 genera of bacteria average worldwide a 13% and severely limit production, quality and safety of food (*1*).

Plant disease control is mainly achieved by treating crops with a vast amount of synthetic chemical pesticides, including fungicides and bactericides. Some of the antifungal and antibacterial compounds, such as azoles or streptomycin, extensively used in plant protection, are used in clinical or in veterinary applications. Therefore, there is a risk that the extensive use in agriculture will increase the probability of selection of resistant strains of human-pathogenic fungi and bacteria. Apart from the environmental and acute toxicity problems posed by several of these pesticides to humans and animals, plant pathogens may become resistant to the active ingredient that compromises its usefulness (*2*, *3*).

Social and political concerns have influenced the practice of crop protection which has been progressively reoriented to a rational use of pesticides and to a reduction of the number of registered active ingredients to more selective, less toxic and with a lower negative environmental impact. As a consequence, several countries have undertaken regulatory changes in pesticide registration requirements. In the EU the regulations on pesticide registration started years ago with Directive 91/414 EC (http://ec.europa.eu/food/plant/protection/index_en.htm) and continued with the new Directive 2009/128/CE.

However, the new scenario of global distribution of plant pests and diseases need for strong efforts in the research of plant protection products to develop novel antimicrobials for agriculture. According to the new rules, such compounds should have good profiles of biocompatibility, biodegradability and low risk of resistance on target pathogens.

Antimicrobial peptides (AMPs) are envisaged as new plant disease control products (*4*, *5*), because of the need for novel antifungals, antibacterials and plant strengtheners that complain more strict regulations about toxicity and environmental impact (cut-off criterium) and are biodegradable. AMPs have been considered as a new generation of antimicrobial drugs that have broad potential application as novel antibiotics to fight resistance appearance in nosocomial infections and opportunistic fungal infections, specially in immunocompromised patients, antibiotics for veterinary use, and biopreservatives for food and other industrial applications (*6–10*).

Natural AMPs are in principle good candidates but are produced in low amounts, some are toxic, low active or unstable, and require generally complex and costly procedures for extraction and purification from the producing organism. Synthetic products offer alternatives but require pharma approaches for development as plant protection products. Therefore, highly efficient, safe and low cost production methods have to be developed for an industrial exploitation in the plant health sector (*5*, *11*).

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

2. Initial Stages in the Process of Antimicrobial Peptide Development

The steps in the discovery and development of AMPs for the plant sector applications are similar to the ones required in the pharma sector for other antimicrobial drugs (Figure 1). New peptides are designed using natural compounds and models and applying structure-function approaches to generate truncated-minimum domain, chimeric, mimetic bounds, or de novo structures. Currently, an initial peptide library is prepared by solid-phase chemistry which is screened for antimicrobial activity against target plant pathogens to search for lead compounds. In a subsequent step, based on the structure of lead compounds, a second library is synthesized by combinatorial approaches by varying selected positions to identify optimized sequences. These optimized leads are screened for additional properties to identify a small collection of selected products for further studies and development. These selected products are subjected to preliminary acute toxicity testing in animal models and to proof-of-concept assays, and eventually may be patented if there is a commercial interest associated. In a more advanced stage in the pipeline, optimization of the synthesis and production costs, development of formulations, and field testing in diseased crops are performed to assure the feasibility and performance of the technology. Finally, industrial exploitation and commercialization may require further tests according to specific country rules, in order to be registered as plant protection products.

2.1. Antimicrobial Peptides from Living Organisms Active against Plant Pathogens

Living organisms secrete a wide range of antimicrobial peptides produced through ribosomal (defensins and small bacteriocins) or non-ribosomal synthesis (peptaibols, cyclopeptides and pseudopeptides).

Microorganisms produce a wide range of antimicrobial peptides that include small bacteriocins and fungal defensins synthesized through ribosomal synthesis, and peptaibols, cyclopeptides and pseudopeptides that are secondary metabolites produced by non-ribosomal synthesis. **Bacteriocins** are secreted by major groups of bacteria, specially by lactic acid bacteria, that kill closely related species (*12*). Bacteriocins of the Class I group are also named lantibiotics and include nisin and subtilin with unusual residues such as lanthionine, methyl-lanthionine, dehydrobutyrine or dehidroalanine. Class II bacteriocines comprises heat stable small non-modified peptides such as plantaricins, pediocins, leucocins and lactococcins), whereas class III includes large peptides of more than 30 KDa which are heat labile. Several filamentous fungi secrete AMPs similar to **defensins** from animals and plants with a compact structure of antiparallel strands stabilized by disulfide bridges (*13*) like the peptides AFP from *Aspergillus giganteus*, PAF from *Penicillium chrysogenum* and *P. nalgiovense* and Anafp from *A. niger* have antifungal activity. **Peptaibols** are produced by several fungi like *Trichoderma* species and are short linear peptides usually with an acyl N-terminus and a C-terminal amino alcohol, containig dialkylated aminoacids (*14*) active against fungi (e.g. Trichokonins Trichorzins and harzianins) and Gram-positive bacteria. C**yclopeptides** are secondary metabolites produced by bacteria, fungi and cyanobacteria with sequences including D- and L-amino acids and special amino acids like allo and diamino derivatives, arranged in a cyclic ring with amide or ester bonds (depsipeptides). Several cyclic peptides incorporate a fatty acid group (cyclolipopeptides) (*15–17*) and have antifungal and antibacterial, cytotoxic and surfactant properties, like the depsipeptides produced by several *Pseudomonas* spp. (e.g. amphisins, corpeptins, putisolvins, syringomycins, syringopeptins, tolaasins and viscosins) (*17*) or the surfactins, iturins, fengycins, polymixins or agrastatins produced by several species of *Bacillus* (*18*). **Pseudopeptides** bear complex amino acid modifications and are produced mainly by bacteria, like the pantocines produced by strains of *Pantoea agglomerans* (*19*), the nucleopeptide derivatives polyoxins, nikkomycins, blasticidin and mildiomycin with antifungal activity (*20*) or the antifungal alanine-epoxycyclohexane substituted dipeptide bacilysin, and the phosphonodipeptide rhizocticin, that are produced by *B. subtilis* strains (*18*).

AMPs from animals range from 12 to 37 amino acids and fit into linear peptides forming extended or helical structures, and cysteine-rich peptides containing single or several disulfide bridges. Linear peptide representatives are melittin from bee venom, cecropins from silk-moth worm, magainins from skin frogs and the extended linear indolicidin from ox. Beta-sheet forming peptides are the insect thanatin and crab tachyplesin, bovine bactenecin, rabbit, porcine and human leukocyte defensins and protegrins (*21*, *22*). Many AMPS of animal origin are active against plant pathogenic microorganisms like cecropin A that is inhibitory to the plant pathogenic bacteria (*23*, *24*), magainin, indolicidin and dermaseptin to *Xylella fastidiosa* (*25*) melittin to the postharvest fungi *Penicillium digitatum* and *P. italicum* (*26*), penaedin to *F. oxysporum, B. cinerea*, and *P. crustosum* (*27*), heliomicin and drosomycin to *Cercospora*, *Fusarium*, *Pyricularia*, *Phomopsis*, *Rhizoctonia*, *Sclerotinia* and *Septoria* (*28*).

Antimicrobial peptides from plants are mainly of the cystein rich disulfide bridged beta sheet peptides. Short peptides are from 30 to 52 amino acids and are grouped into thionins, plant defensins, heveins and knottins (*29*, *30*). AMPs of plant origin are also inhibitory to plant pathogens. Thionins are active against species of plant pathogenic bacteria (*31*), and to the fungi *Thielaviopsis basicola*, and *Drechslera teres* (*32*), *F. oxysporum*, *P. cucumerina*, and *B. cinerea* (*31*), and radish defensins are inhibitory to *Pyrenophora tritici-repentis* (*33*) and *Fusarium culmorum* (*34*).

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

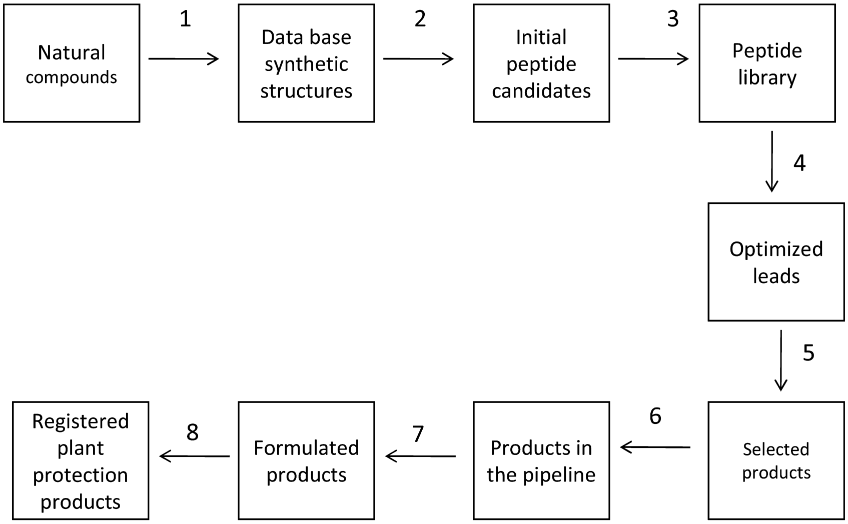

Natural compounds
1
Data base synthetic structures
2
Initial peptide candidates
3
Peptide library
4
Optimized leads
5
Selected products
6
Products in the pipeline
7
Formulated products
8
Registered plant protection products

Figure 1. Flow diagram of the development of antimicrobial peptides as plant protection products for disease control. New peptides are designed from natural compounds using structure-function approaches and generating truncated-minimum domain, chimeric, mimetic bounds, or de novo structures (1). The new peptides are synthetized by solid-phase chemistry to made an initial peptide library (2). The initial peptide library is screened for antimicrobial activity against target plant pathogens to search for lead compounds (3). Lead compounds are used to design new peptides usually by combinatorial approaches of selected amino acid positions and made an optimized peptide library (4). The optimized leads are screened for additional properties based on animal and plant cytotoxicity and protease susceptibility to made a small collection of selected products for further studies (5).These products are subjected to preliminary acute toxicity testing in animal models and to proof-of-concept assays, and may be further patented (6). Optimization of the synthesis and production costs, development of formulations, and field testing in diseased crops are performed (7). Further tests are prepared according to specific country rules to registration and commercialization (8).

2.2. Design of Synthetic Derivatives

Traditionally, the search for novel AMPs involves the identification of active peptides from natural sources (*21*, *22*, *29*, *35–39*).

Such studies are followed by the design of synthetic peptide analogues with the aim of delineating the structural requirements for selective antimicrobial activity as well as of analyzing structure-activity relationships. Much research within this area has been focused on designing short analogs with increased antibacterial activity and low cytotoxicity against mammalian cells. To control peptide selectivity, several physicochemical parameters need to be addressed including net charge, helicity, hydrophobicity per residue (H), hydrophobic moment (μ), and the angle subtended by the positively charged polar helix face (Φ) (*40–42*). Numerous studies support that the activity of AMPs depends primarily on their overall physicochemical properties, rather than their precise amino acid sequence.

In particular, the synthesis of AMPs obtained by modification of known natural sequences such as cecropins, magainins, melittin, indolicidin or temporins is one of the most prominent approaches for the discovery of active peptides for plant protection (*4*, *5*, *11*). These modifications include the addition, deletion or replacement of one or more residues, the truncation of N- or C-terminus, and the assembly of segments from different natural peptides. These peptide analogues showed reduced toxicity compared to their natural parent sequences, while displaying high activity against phytopathogens. Moreover, the juxtaposition of fragments of these natural peptides resulted in chimeras with improved biological properties. Among them, cecropin-melittin hybrids have been the most studied either in agroscience or in biomedical fields (*4*, *43*, *44*).

De novo design of antimicrobial peptides maintaining the crucial features of native antibacterial peptides (charge and amphipathicity) has led to the development of compounds with remarkable activity. It is worth to note the minimalistic *de novo* approaches to design model amphipathic helical peptides composed of repeating residues or primary sequences (*45–47*). For instance, peptides composed of solely of lysine and leucine residues have been characterized as possessing strong antimicrobial activity (*42*, *48–52*). This strategy has been also applied for the de novo design of cyclic antimicrobial decapeptides of the BPC series that departed from an initial library of 4 to 12 amino acid components and focused finally in a slighly active decapeptide that was further optimized using a combinatorial approach (*53*, *54*).

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

2.3. Screening for Activity

One of the most important steps in the development of new peptides is the platform used to evaluate their activity that should be reproducible, use minimum amounts of product and allow testing large amounts of products, specially when combinatorial approaches are involved (*55*). Most methods focus on determining the activity in growth inhibition. Tradicional methods used diffusion tests in solid medium, in which knowm amounts of peptide are deposited in the agar medium where the target microorganism has been seeded as an overlay layer.This method is highly dependent on the solubility, diffusion and binding properties of the peptide to the media components, that are usually not well known during the screeening of the peptide library. An alternative is the liquid assay consisting of mixing known amounts of the AMP with the pathogen cells in microplate wells and monitoring growth with an automatic reader. This method is highly dependent on solubility of the peptide as well as the type of growth of the target pathogen (tendency to sediment or form filaments). Another method used consist of a contact test that is based on the same principle than the liquid growth inhibition assays but after a given period of incubation surviving cells are determined. In fact this assay permit to determine the microbiocidal activity of the AMP, and is suitable in more adavanced studies.

The developmental stage of target cells (vegetative cells, spores) has also an influence. Upon a proper selection of the concentrations, the Minimal Inhibitory Concentration (MIC) can be calculated, as well as the Median Effective Concentration (ED50) which permit to compare different peptides. In the case of the contact test and departing from the kinetics of survival the Decimal Reduction time (D) can be calculated as in temperature killing assays and this assay can be used to differentiate peptides having similar MIC or ED50 values that below 10 μM are considered as good. Several peptides that show a potent bactericidal effect against *E. amylovora* have D values at concentrations around the MIC of less than an hour, similar to reports with peptides of 12 to 20 amino acids against *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (*52*).

In the growth inhibition assays the activity is strongly dependent on medium characteristics and specially ionic composition and strengh and the presence of certain organic products components negatively charged may give abnormal low activities. For example, when the hexapeptide PAF26 was tested against *P. digitatum* in 10-to-20 fold diluted PDB broth it showed a MIC of 4-6 μM, whereas in undiluted broth the peptide was lees active and the MIC increased to 20-40 μM (*26*).

In some development approaches using combinatorial chemistry, the lead optimization step requires the screening of several hundreds or thousands of compounds. In these cases, High Throughput Screening Systems (HTS) based on cell viability analysis using vital staining or fluorescence or luminiscence reporters are used. The degree of permeation of bacterial and fungal cell membranes can be used based on dyes that are able to penetrate only damaged membranes, and bind to intracellular targets or are degraded in the cytoplasm. These methods can be performed in multi-well plates and are reliable to be automated. An example is the fluorescent dye SYTOX Green, that only penetrates cells when plasma membrane integrity is compromised (*56*). Peptides that act through membrane permeabilization allows the penetration of SYTOX Green into the target cell, producing an increase in fluorescence upon binding to DNA. This methodology has been tested with good results in Gram-positive bacteria, like *Streptococcus mutans* (*57*) and also in Gram-negative bacteria as *Escherichia coli* and *P. aeruginosa* (*58*) . In our laboratory, this methodology has been proven against several Gram-negative bacteria, like *Erwinia amylovora* and *P. syringae* and *Penicillium expansum* for evaluating a collection of synthetic linear undecapeptides (*59*).

A similar methodology is based in Berberine, that fluoresces when bind to DNA and glycosaminoglycans (*60*) and has been used for measuring bacteriocin activity against *Listeria inoccua* and *B. cereus* (*61*). The activity of α-defensin and several cationic peptides have been studied against *E. coli* (*62*) and Gram-positive bacteria (*63*) using 2-nitrophenyl β-D-pyranoside (ONPG) that follows a different principle and once penetrates the bacterial cell cytoplasm, it is hydrolyzed by β-galactosidase, and converted to ONP that is determined colorimetrically. Another approach uses 3,3'-dipropylthiacarbocyanine (diSC$_3$-5), a membrane potential-

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

sensitive cyanine dye and has been used to determine the mechanism of interaction of cationic antimicrobial peptides with cytoplasmic membrane of *E. coli* (*64*) or in *Streptococcus mutans* (*57*) .

In many cases a good correlation has been found between SYTOX green fluorescence and antimicrobial activity though in other cases this correlation is not so good, thus peptides with low permeabilization activity and high antimicrobial activity and also with high permeabilization activity and low antimicrobial activity has been observed. This agrees with other studies that have argued that there is not always a complete correlation between cell permeation and antimicrobial activity (*64*, *65*).

Some peptides kill bacteria and fungi, but they either do not disrupt bacterial membranes or have additional modes of action in intracellular targets (*66*). In these cases, engineered target pathogens can be constructed expressing *gfp* or *lux* genes, and used to screen AMP libraries on the basis of fluorescence increase or bioluminiscence decrease. These reporters have been used for standard antibiotic susceptibility analysis in clinical pathogens (*67*) and massive screeening can be performed faster than with absorbance, and using microplate readers. However, these methods are highly dependent on the success of producing labelled target pathogens, and specially in the case of *gfp* technology the best results are obtained with unstaible *gfp* mutants.

As in many functional peptides, biochemical and biophysical cellullar processes affected by AMPs can be used as target. Although most AMPs act through a membrane damage mechanism, several peptides interfere with cellular processes. Binding assays have been used for analyzing AMPs interaction with bacterial lipopolysaccharides or lipotheicoic acids (*68*) , ergosterol (*16*) , DNA or RNA (*69*) or chitine synthesis (*70*).

When assaying anitmicrobial activity of peptides in that the mechanism of action requires membrane penetration, the electrostatic attraction between peptide and cell membrane is perturbed by the increase of ionic strength and peptide antimicrobial activity may diminish with the addition of salt ions to the *in vitro* assay medium.

In spite that most peptides capable of controlling plant pathogen infections have a mechanism of action direct towards the target pathogen, a few peptides act as effectors of the adaptive immune system in animals and humans (*7*) or have been reported as elicitors of plant defence response (*71–73*). therefore, new methods of screening peptide libraries are necessary in these cases. The most common way to study plant defence induction by peptides is through analyzing mRNA expression of gene markers like *pal1*, *prx* and *hpl* in cucumber; *UBQ10*, *PR1*, *PR2*, *PR5* and *vps2* in *Arabidopsis* (*71*) or *PR-1* and *pid-2* in tomato (*74*), using RT-PCR. Also models based on plant cell culture of *Arabidopsis*, tomato and tobacco has been proposed to study the induction of defense responses with these molecular tools (*75*) or the induction of alkalinisation in cell suspension cultures exposed to nanomolar concentrations of the peptides (*76*). These method has been used with tobacco BY-2 cells to assess the eliciting properties of synthetic ultrashort cationic lypopeptides in (*71*) or flagellin-derived peptides (*77*). Instead of directly measure the pH several colorimetric and fluorescent dyes can be used to record pH changes of medium like bromothymol blue, a colorimetric pH indicator, and fluorescein, a

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

fluorophore with dependent fluorescence on its ionization state. Thus, fluorescence in function of pH allow to use micro-well plates to perform alkalinisation assays making this methodology easy to use and properly to be used as a high-throughput platform in a microplate reader. This method has been tested in our laboratory with synthetic linear undecapeptides in tobacco BY-2 cells, observing a differential behaviour among peptides. Recently, a new method has been proposed based in a colorimetric assay for quantifying the generation of H_2O_2 in plant cells cultures exposed to elicitors based on the dye DA-64, and a horseradish peroxidise assay using a microplate reader (*77*).

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

2.4. Screening for Additional Key Properties

The citotoxicity of AMPs to eucaryotic non-target cells is an important issue because it can be related to adverse effects to animals and plants. It is also important to have information on stability of the activity of the peptides in the presence of tissue fluids and cell components, specially to proteases due to its peptidic nature.

In vitro toxicity assays are necessary for screening optimized leads before performing animal or plant toxicity assays that do not permite massive screening of compounds. Basic toxicity is generally tested using animal cells (red blood or mammal cell lines). The most common test is the hemolytic assay using hemoglobin release as a lysis indicator, that can be automatized in a microplate reader assay. However, results are affected by the type of RBC used (human, sheep, horse). For example, sheep erytrocites are less sensible than rabbit and human (*78*). Methods based on mammalian cell lines (HL-60; McCoy, L-929, K-562, HOK-16B) use different tetrazolium salt colorimetric indicators as reporters (*79–82*). Citotoxycity in plant cell material have been developed for other pesticides different from AMPs but can be adapted easily. Tobacco BY-2 cell cultures can be used coupled to the measurement of cell viability using vital staining or alcalinization/acidification chromophores or fluorochromes, but it is difficult to differentiate cell damage from cell responses related to plant cell defence (*71*, *83*). Another techniques involve pollen germination of apple or tomato flowers (*84*), necrosis in tobacco leaf mesophyl after infiltration or phytotoxicity in *Arabidopsis thaliana* plants (*85*).

The stability of AMPs to protease digestion is an important property for assuring a reasonable shelf-life of the peptides in the environment where will be applied (*86*). However, the susceptibility to proteases is on one hand negative because it compromises peptide activity but in another hand it can be considered positive at the time of low persistence in the environment, a desirable property for a modern pesticide. Current *in vitro* methods consists of using different proteases or protease cocktails, or natural fluids (e.g. saliva, plasma, leaf extracts) (*82*, *87*), and monitoring hidrolysis by HPLC-mass spectrometry or SDS-PAGE gel electrophoresis (*82*, *85*, *88–90*) .

Although proteases are key factors in determining the activity in natural fluids, other natural compounds can have an influence in activity like phenolics and polyphenolics (*91–93*). Also, metal cations and anions have been reported to affect the activity of some AMPs, as in the case of the anionic AMP kappacin with increased activity by ZnCl2 salts (*94*).

2.5. CECMEL11 and CYC10 Chemical Libraries

We designed 22 undecapeptides (CECMEL11) derived from WKLFKKILKVL-NH_2 (Pep3), an 11-mer peptide corresponding to cecropin A(*2-8*)-melittin(*6-9*) (*95*, *96*), and evaluated them against plant pathogenic bacteria such as *Xanthomonas axonopodis* pv. *vesicatoria*, *Pseudomonas syringae* pv. *syringae* and *Erwinia amylovora* (*24*). The design was based on the structural parameters required for antimicrobial activity. When Pep3 is represented as an ideal α-helix by means of an Edmunson wheel plot, its amphipathic character becomes evident (Figure 2). Thus, we investigated the replacement of the amino acids located at the interface with residues with various degrees of hydrophilicity and hydrophobicity, as well as the blocking of the N-terminus. Analysis of the antibacterial activity showed that structural features that seem to be important are a basic N terminus and a hydrophobic C terminus. Moreover, the evaluation of the antibacterial activity of N- and C-terminal deletion analogues as well as of C-terminal acid derivatives pointed out that the entire sequence of Pep3 is necessary for its full activity and that a C-terminal amide is required.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

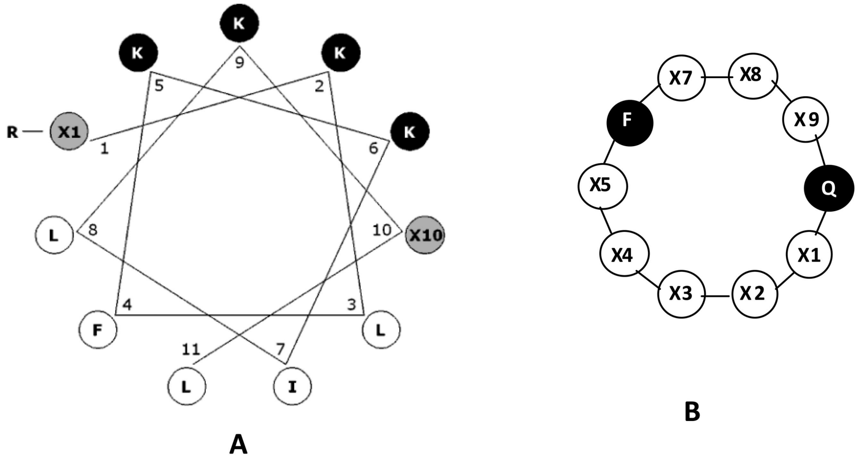


*Figure 2. Strategy for lead optimization of linear undecapeptides and cyclic decapeptides using a combinatorial approach on specific residue positions. Design of the CECMEL11 (**A**) and CYC10 (**B**) peptide libraries. **A)** Black background stands for hydrophilic amino acids and white background for hydrophobic amino acids. The CECMEL11 library was designed by combining: R= H, Ac, Ts, Bz or Bn; X1= Lys, Leu, Trp, Tyr or Phe; X10= Lys, Val, Trp, Tyr or Phe. **B)** The CYC10 library was designed by combining: X1-X9= Lys or Leu.*

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Other factors that are important in designing AMPs are the toxicity and the stability to protease degradation. Since the mechanism of action of AMPs consists of cell membrane disruption, toxicity to animal or plant cells (cytotoxicity) may be a problem. Thus, we evaluated CECMEL11 peptide cytotoxicity against human red blood cells and we observed that it was also strongly influenced by the nature of the amino acid replacement in Pep3 (*24*). In accordance with previous studies on AMPs, an increase in the peptide hydrophobicity was related to an increase in cytotoxicity (*55*, *87*). This selectivity has been attributed to the differences in membrane lipid composition between bacteria and mammalian cells. The absence of acidic phospholipids and presence of sterols reduce the susceptibility of eukaryotic cells to lytic peptides (*97*). On the other hand, protein digestion stability is a desired property in AMPs to assure a reasonable half-life of the molecules in the plant environment. Proteases from epiphytic microorganisms or intrinsic to the plant in internal tissues may degrade antimicrobial peptides (*95*, *96*). Again certain replacements of amino acids in Pep3 had a strong influence in susceptibility to protease digestion (*24*). From this study we were able to identify one peptide, KKLFKKILKFL-NH_2 (BP76), with improved antibacterial activity and minimized cytotoxicity and susceptibility to protease degradation compared to Pep3. With the aim of obtaining a set of BP76 analogues with in vitro activity, we prepared a 125-member CECMEL11 peptide library using a combinatorial chemistry approach (*98*). The library was designed by combining five variations at each R, X1 and X10 positions: R = H, Ac, Ts, Bz or Bn; X1 = Lys, Leu, Trp, Tyr or Phe; X10 = Lys, Val, Trp, Tyr or Phe. This library was evaluated for antibacterial activity against *E. amylovora*, *X. axonopodis* pv. *vesicatoria*, and *P. syringae* pv. *syringae*, for antifungal activity against *Penicillium expansum* (*59*), and for hemolytic activity and proteolytic susceptibility. A view of the antibacterial and hemolytic activities for a set of 50 members of the library is shown in Figure 3. We identified a set of 15 peptides displaying high antibacterial activity and another set of 15 peptides exhibiting high antifungal activity. The two sets had four peptides in common, with both high antibacterial and antifungal activities. We found that the CECMEL11 peptides with the highest antibacterial activity share the following structural features: a net charge of +4 to +6, a Lys at position 1, and an aromatic residue at position 10. Moreover, N-terminal derivatization led to less active peptides. In contrast, different structural requirements were associated with high antifungal activity against *P. expansum*: a charge of +4 or +5, a Val at position 10, and an Ac group at the N-terminus. Based on the results obtained in this study, the different activity and specificity profiles of the CECMEL11 peptides between pathogens can be attributed to differences in the membrane components of the target microorganism, e.g. charge and lipid composition, which would influence rates of binding of cationic peptides to the membranes. On the other hand, we observed that the less hemolytic peptides incorporated a Lys residue either at position 1 or 10. N-terminal derivatization increased the hemolytic activity and peptides containing a Trp were the most hemolytic. Peptides with an optimal balance between antibacterial and hemolytic activities were identified (*98*). In contrast, the most active peptides against *P. expansum* were significantly hemolytic (*59*). However, these hemolytic activities are similar to that observed for the reference fungicide imazalil. In addition, peptides with an optimal biological activity profile also showed good stability towards protease degradation.

Peptide stability against protease hydrolysis can be increased by the development of synthetic analogues with similar structural features but containing nonproteinogenous amino acids. In particular, incorporation of D-amino acids is an approach used to protect peptides against enzymatic hydrolysis, since only a few enzymes are known to digest amide bonds involving D configuration (*99*). This strategy has been used to improve the biological activity profiles of synthetic antimicrobial peptides, not only increasing the resistance to proteolytic enzymes but also reducing the hemolytic activity while maintaining the antimicrobial activity (*42*, *81*, *82*, *100–103*). To improve the activity of one of the best antibacterial peptides, KKLFKKILKYL-NH_2 (BP100), identified from the CECMEL11 library, we synthesized 31 derivatives incorporating D-amino acids into the sequence (*92*). Besides testing the influence of replacing all-L amino acids on antibacterial and hemolytic activity, the influence of incorporating 1 D-amino acid, 2 or 3 D-amino acids at adjacent or nonconsecutive positions, and 4 to 10 D-amino acids at either the N or the C terminus was also investigated. D-Diastereoisomers exhibited complicated antibacterial activity patterns and did not display a simple dependence on the polarity of the residue. However, the introduction of D-amino acids at the N or C terminus resulted in distinctly improved activity. Peptides containing D-amino acids at the C terminus were more active, and their activity increased with the number of D-amino acids. The all-D isomer resulted to be the most active sequence. Regarding the hemolysis, among the D-isomers, only five sequences were more hemolytic than BP100. Moreover, all peptides were more stable against protease degradation than the parent peptide.

Another strategy to improve the metabolic stability of peptides is by reducing the flexibility of the secondary structure through cyclization, either by disulphide or lactam bond formation. Apart from limiting the accessibility to proteases, peptide cyclization has been shown to promote an increase of the selectivity for bacteria versus mammalian cells, leading to a decrease in the hemolytic activity (*102*, *104–106*). In fact, cyclization results in a suitable amphipathicity to enhance electrostatic interactions in initial binding with the negatively charged membranes of bacteria, so that interactions with the electrically neutral lipids of the membrane of red blood cells are reduced. Within our current research focused on identifying peptides active against the plant pathogenic bacteria *E. amylovora*, *X. axonopodis* pv. *vesicatoria*, and *P. syringae* pv. *syringae,* we synthesized cyclic peptides of 4-10 residues consisting of alternating cationic and hydrophobic amino acids with general formula c(X_n-Y-X_m-Gln) where X is Lys or Leu, Y is L-Phe or D-Phe, m=n=1, or m=3 and n=0-5 (*53*) . The cyclic decapeptide c(KLKLKFKLKQ) (BPC16) was active against *P. syringae* pv. *syringae* and *X. axonopodis* pv. *vesicatoria,* but it was not active against *E. amylovora* and displayed a high hemolytic activity. In order to improve these properties, a library (CYC10) was designed based on the sequence of BPC16 and comprised 56 cyclic peptides (*54*). The sequences incorporated a Phe and a Gln residue at positions 6 and 10, respectively. The other positions consisted of all the possible combinations of three Leu and five Lys. The CYC10 library was screened for antibacterial

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

activity and eukaryotic cytotoxicity, and led to the identification of peptides with improved activity against *P. syringae* pv. *syringae* and *X. axonopodis* pv. *vesicatoria*. Moreover, peptides active against *E. amylovora* were found. Best peptides of this library incorporated the substructure K^5FKLKQ^{10}. To check the influence on antibacterial and hemolytic activities of residues at positions 1 to 4, a second library was designed by using DOE. This library included 16 sequences containing at positions 1 to 4 all possible combinations of Leu and Lys, and the substructure K^5FKLKQ^{10}. The activity against *E. amylovora* was further improved and the best peptides displayed a low eukaryotic cytotoxicity at concentrations 30-120 times higher than the MIC values. The DOE permitted to define rules for high antibacterial activity and low cytotoxicity, being the main rule $X^2{\neq}X^3$, and the secondary rule X^4= Lys.

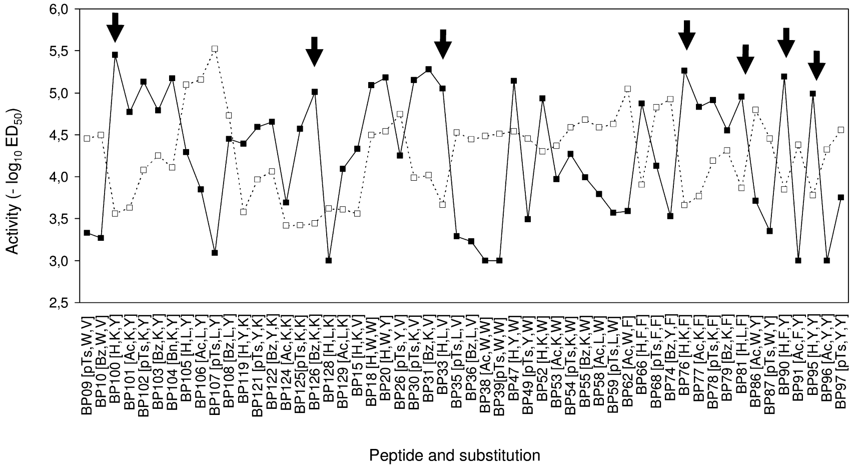


Figure 3. Optimization of peptide properties. Antibacterial activity against Erwinia amylovora (black symbols) and hemolytic activity (white symbols) for a set of linear undecapeptides obtained after the combinatorial procedure consisting of substitutions at the R, X1 and X10 positions. Suitable compounds exhibit high antimicrobial activity and low hemolytic activity.

2.6. Mechanism of Action of the CECMEL11 and the CYC10 Peptides

Most AMPs have cationic charge and amphipathic arrangement that enable their interaction with biological membranes resulting in many cases in cell membrane disruption and cell death. Many studies propose this membrane disruption as the main mechanism of action of AMPs (*107*, *108*) though other studies suggest that AMPs have other intracellular targets such as enzymes or as DNA (*7*, *8*, *66*, *109–112*). However, the exact mode of action of these peptides is poorly understood. Elucidation of their mechanism of action and their specific membrane damaging properties is crucial for the rational design of novel antibiotic peptides with high antibacterial activity and low cytotoxicity.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

The use of microscopy to view the effects of antimicrobial peptides on microbial cells has helped to identify general target sites. For example, confocal laser-scanning microscopy has shown how magainin 2 binds to the cell surface, whereas buforin II enters the cell and accumulates in the bacterial cytoplasm (*52*). Scanning and transmission electron microscopy have been used to demonstrate the damaging effects of antimicrobial peptides such as SMAP29 that induced ultrastructural damage in the gram negative bacteria *Pseudomonas aeruginosa* (*113*) or CP11CN that produced cell wall breaks and variability in wall thickness in the gram positive bacteria *Staphylococcus aureus* (*63*).

Peptides capability to interact and destabilize lipid bilayers has been has been studied in artificial lipid membranes using structural and biophysical techniques (*114*). Assessing the interaction of antimicrobial peptides with phospholipids in model membranes to provide insights into mechanisms of activity might be more relevant than using electron microscopy to determine the type of cellular damage induced by peptides. Techniques such as X-ray crystallography, NMR spectroscopy and Fourier transform infrared (FTIR) allow determining attraction, attachment, insertion and orientation of the peptide, as well as, the thickness and the integrity of the lipid bilayer (*36*). Another method based on these model membranes is the carboxyfluorescein leakage assay. In this method, carboxyfluorescein-loaded liposomes are used. Liposomes lysis produces carboxyfluorescein leakage that can be measured as an increase of fluorescence emission. This method has been used to test the effect of lipids composition in the permeabilization of liposomes by α-defensin cryptdin-4 (*62*) and also by series of short cationic amphiphilic peptides derived from sapecin B (*115*).

Antimicrobial peptides have been long considered to play a key role in plant defence, both as part of pre-existing, developmentally regulated defence barriers and as components of the defence responses induced upon infection. Cyclic lipopeptides, such as surfactins and fengycins from the bacterial plant biocontrol agent *Bacillus subtilis* (*73*) or massetolide A from *Pseudomonas fluorescens* SS101 (*116*), have been shown to stimulate the induction of ISR in tomato plants. Other studies focused on synthetic ultrashort cationic lipopeptides also have provided new evidences that these peptides, similar to *Bacillus subtilis* cyclic lipopeptides, are capable of inducing defence signalling pathways in plants and systemic protection to foliar bacterial and fungal disease in cucumber and *Arabidopsis* plants (*71*). Some studies suggest that membrane perturbations in plants by peptides could activate signalling cascades leading to plant defence activation due to changes in membrane potential which are described as the initial responses in several signalling pathways (*72*).

Several AMPs have cell penetrating capabilities in non-host cells, and this property has been shown mainly in mammalian and plant cells (*117*). For example, BP100 (KKLFKKILKYL-amide), originally designed as an antimicrobial peptide against bacterial plant pathogens, has been reported as a fast and efficient cell-penetrating agent to deliver functional cargoes, like the actin-binding Lifeact peptide (MGVADLIKKFESISKEE) into tobacco cells (*118*).

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Taking these observations into account, biophysical studies were employed to explore the mode of action of KKLFKKILKYL-NH_2 (BP100), one of the lead peptides identified from the CECMEL11 peptide library (*119*). These studies took advantage of the intrinsic Tyr fluorescence of this peptide to explore its binding affinity and damaging effect on phospholipid bilayers having lipid compositions similar to that of the bacterial cytoplasmic membrane. As indicated by the high partition constants, BP100 showed a stronger selectivity toward these anionic bacterial membrane models. For these anionic systems, membrane saturation was observed at high peptide/lipid ratios and it was related with BP100-induced vesicle permeabilization, membrane electroneutrality, and vesicle aggregation. BP100 translocation was also unequivocally detected. In addition, the peptide concentrations required for saturation and neutralization were in the range of that required for microbial growth inhibition (*98*).

The mechanism adopted by BP100 in disrupting the Gram-negative *Escherichia coli* bacterial envelope was also explored (*120*). Standard antimicrobial activity assays and zeta potential studies demonstrated a clear correlation between the MIC and the corresponding alterations in the *E. coli* surface charge. Specifically, neutralization of the bacterial surface was detected when treating *E. coli* with BP100 concentrations close to MIC values. Atomic force microscopy (AFM) was used to visualize the structural effect of the interaction of this peptide with the *E. coli* cell envelope and illustrated the time- and concentration-dependent antimicrobial action of this peptide. These results point out that a critical AMP concentration, equivalent to MIC values, is necessary for *E. coli* membrane disruption to occur.

A combined Molecular Dynamics (MD) and biophysical study was undertaken to investigate the interaction of the cyclic peptide c(KKLKKFKKLQ) (BPC194), one of the best antibacterial peptides identified from the CYC10 peptide library, with anionic lipid bilayers (*121*). The linear analogue, KKLKKFKKLQ (BPC193), which was inactive against Gram-negative phytopathogenic bacteria, was also studied to contrast the behaviour of these two related peptides and extract the molecular basis for antimicrobial activity. This study showed that the cyclic peptide BPC194 bound stronger to negatively charged membranes than the linear analogue BPC193, folded at the membrane interface and adopted a β-sheet structure characterized by two turns. Subsequently, BPC194 penetrated deeper into the bilayer while the linear peptide BPC193 remained essentially unfolded at the surface. The molecular basis for the enhanced activity of the cyclic sequence can be related with the restricted number of conformations available for the cyclic peptides. The linear peptide BPC193 has large conformational entropy, and backbone folding is less favourable than in the cyclic analogue BPC194. In contrast, BPC194 can adopt a favourable orientation towards the membrane and acquire an ordered structure with the lysine residues on opposite strands aligned. This structure leads to a high charge density and an amphipathic arrangement, which allows the cyclic peptide to locate itself deeper in the membrane as well as to perturb it more than the linear analogue. These results can be used as a guideline for design of novel antimicrobial peptides.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Table 1. Effect of the linear undecapeptides BP100 (KKLFKKILKYL) and BP143 ((KKLFKKILKYL) on the severity of infections by *Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae* on pear, and *Xanthomonas axonopodis* pv. *vesicatoria* on pepper in comparison to a non-treated control and the reference antibiotic streptomycin. The assays were performed in detached flower and immature fruit, and in whole plants. The underlined letter in the sequence of a peptide means a D amino acid substitution

Treatment	*Plant pathogen system*													
	E. amylovora-pear						*X. a. pv. vesicatoria-pepper*				*P. syringae pv. syringae-pear*			
	Flower		*Immature Fruit*		*Whole plant*		*Leaf*		*Whole plant*		*Leaf*		*Whole plant*	
Non-treated	76	a	72	a	79	a	94	a	100	a	85	a	97	a
BP100	48	b	48	b	28	b	61	b	76	b	59	b	50	b
BP143	41	b	43	b	26	b	67	b	37	c	63	b	22	c
Streptomycin	19	c	14	c	17	b	17	c	13	d	7	c	0	d

Data were taken from Badosa et al. (*98*) and Güell et al. (*92*). Severity values correspond to a relative scale from 0 to 100% of the maximum attained infection on each experimental system.Means with the same letter within an assay do not differ significantly ($P<0.05$) according to Waller-Duncan test.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Table 2. Activity, selectivity and toxicity of selected antimicrobial linear undecapeptides

			Antimicrobial activity[a]				*Hemolytic*[b] *activity*	*Protease*[c] *stability*	*Toxicity*[d]
Peptide	*Sequence*	*Properties*	*Food-borne bacteria*	*Plant pathogenic bacteria*	*Animal pathogenic Gram-positive*	*Fungi*			
BP100	KKLFKKILKYL	antibacterial G-	+++	+++	+-	-	l	m	vl
BP18	WKLFKKILKWL	antibacterial wide	+++	+++	+++	-	m	m	nt
BP21	Ac- FKLFKKILKVL	antibacterial G+ and antifungal	+	+	++	+++	m	m	vl
BP15	KKLFKKILKVL	antibacterial G- and antifungal	++	+++	-	+++	l	m	vl

[a] Minimum concentration of peptide inhibiting growth (MIC). The inhibition assay was performed in cell/spore suspensions in liquid media; Luria-Bertani (LB) for bacteria and Potato-dextrose (PD) for fungi. The assay was performed at the original medium concentration. +++, <6.25; ++, 6.25-12.5; +, >12.5-25; -, >25 µM [b] Human red blood cells assay at 150 µM. l, <10%; m, 10-25%. [c] Resistance to hydrolysis in Proteinase K degradation assay test at 60 min. [d] Minimum lethal oral dose in mice . vl, higher than 1000 mg/g animal weight; nt, not tested.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

3. Development of Products from the Pipeline

3.1. Proof-of-Concept

In vitro tests are not always predictive of the capacity of the AMP to inhibit pathogen infection on host. Host (phenological stage, plant organ or tissue) and pathogen components (way of inoculation of the pathogen, spores or vegetative cells), strategy of treatment (preventative, simultaneous or postinfection application; wounded or unwounded tissues) and environmental factors (temperature, wetness) can influence the activity of AMPs. Therefore, assays on plant material are required, and the first stage of these assays are based on *ex vivo* methods with detached plant parts that are not equivalent to whole plant assays, but are close and facilitate massive screening procedures.

Several *ex vivo* systems have been reported in evaluation of antimicrobial peptides based on detached plant organs like leaves, flowers, fruit and roots (*26*, *59*, *85*, *92*, *95*, *98*). Most reports involving AMPs have used this approach, although it is not predictive of what happens with the whole plant due to possible responses by the host, and whole plant assays are required and considered the most suitable for an AMP development. When, massive screening is necessary peptides can be tested in *Arabidopsis thaliana*, a plant model system which is susceptible to several plant pathogens including bacteria and fungi (*122*, *123*). If massive screening is not necessary, whole plant assays can be done in representative plant pathosystems. For example, ultrashort cationic peptides have been tested in *A. thaliana* to control *P. syringae* (*85*), linear undecapeptides have been assayed on pear plamts to control *E. amylovora* and *P. syringae* pv. syringae and in *X. axonopodis* pv. vesicatoria in pepper plants (*92*). In these assays higher doses of peptides are required, generally from 25 to 100 times higher than the *in vitro* MIC values (50 to 200 µM), and only a few AMPs are tested compared to reference compounds. The best peptides from the CECMEL11 and CYC10 libraries are comparable in terms of activity to antibiotics, such as streptomycin, used in agriculture for bacterial disease control with an *in vitro* activity of 2-9 µM and operational doses for field treatment of around 100-200 µM. An overview of the performance of the lead linear undecapeptides from the CECMEL11 library, BP100 (all L- amino acid) and BP143 (one D- amino acid diaestereomer) is presented in Table 1.

When performing *in vivo* assays (*ex vivo or in planta*), several factors have to be taken into account. Plant fluids decrease the activity of antimicrobial peptides, as it has been reported in tomato or tobacco where plant protein extracts or intracellular fluids strongly decreased the activity of antimicrobial peptides (*96*) or in pear where activity of various linear undecapeptides against *X. axonopodis*, *P. syringae* and *E. amylovora* is reduced in leaf extracts in comparison to the activity observed in buffer (*92*). Also the presence of wounding can have an effect on peptide activity, thus peptide BP100 has shown greater activity against *E. amylovora* in pear and apple flowers (*98*), where wounding are not required for pathogen infection, but wounding is required in immature pear fruits (*92*).

The way of application is another important aspect. Several strategies have been proposed, as the preventive application, when peptide is applied in the plant or tissue before pathogen inoculation; the co-inoculation, when peptide and

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

pathogen are applied at the same time; or the pre-incubated inoculation, when peptide is mixed with the pathogen and applied after an incubation time. In many reports, *in vivo* tests are performed by inoculating a mixture of the pathogen cells and the peptide into the plant material either immediately or pre-incubated (*23*, *95*, *108*). In contrast, other reports, apply AMPs preventatively into wounds or on plant surfaces, and subsequently inoculate the pathogen into the treated site (*88*, *92*, *98*). Obviously, in the first approach, the peptide has more chance to interact with the target, leading to a dramatic decrease of the number of alive pathogen cells that are inoculated and, thus, giving disease overcontrol. Contrarily, the second approach is closer to the real situation because peptide can interact with plant tissues (inactivated by plant fluids or degraded by proteases) before pathogen arrival. Therefore results using both methodologies are not comparable.

As far as we know, and unfortunatelly, there are no field tests reported on the use of antimicrobial peptides for plant disease control. In our experience, the amount of product for a minimal experimental plot needed (aroung 10 g) and the associated cost is a limiting factor. However, several field trials are under process in our laboratory.

3.2. Toxicology

AMPs from natural sources (e.g. microbial, animal or plant origin) or synthetic can be toxic to animals or plants, and this property should be detected before proceeding to further development stages. Some indication of the potential toxicity is provided by the *in vitro* citotoxicity tests (hemolytic, plant cell lysis or necrosis). However, whole plant or animal toxicity tests have to be performed according to the experience in the pharma sector. Since the main way of interaction of AMPs with animals and humans when used as plant protection products is by inhalation, dermal or oral ingestion through food, acute toxicology is of priority. Generally, these tests are delayed to an advanced development of AMPs in the pipeline due to the cost of the test itself but also to the cost of the amount of peptide needed (e.g. more than 2 g).

In animal models, the most common toxicological tests are acute oral toxicity in mice or rat, to assess the median lethal dose (LD_{50}) and the lower limit lethal dose (LLD) (OECD Test Guidelines). Intraperitoneal injection (also named abnormal toxicity test) is recommended, that in development of AMPs for the human or veterinary sectors can be combined with inoculation of a model bacterial pathogen (e.g. *E. coli* pathogenic strains, *S. aureus*, etc.). Complementary tests to evaluate other ways of interaction are dermal, eye and inhalation irritation tests in guinea pig or rabbit. Unfortunatelly, in spite of the great number of reports on AMPs existing in the litterature, there is a lack of information on toxicity in whole animal tests for most AMPs.

Toxicity in whole plant models can be performed at small scale level using *Arabidopsis thaliana* plantlets, or tobacco leaf infiltration assays, as well as detached flower, fruit or leaf assays as for other fungicides and bactericides for plant protection products (*85*).

Some of the peptides from the CECMEL11 and CYC10 libraries have been submitted to oral acute toxicity testing in mice. Generally, LLD and LD_{50} for

BP100 and BP76 (bactericidal), BP22 (fungicidal) and BP15 (wide spectrum) is higher than 1,000-2,000 mg/Kg of body weight, thus it is considered of very low oral toxicity (*11*).

3.3. Optimization of Synthesis and Production Costs

An industrial exploitation of antimicrobial peptides requires mass production, which can be accounted through chemical or quimioenzymatic synthesis, or by means of microbial or plant biofactories.

Chemical synthesis using solid or liquid phase protocols, or enzymatic procedures is only economically feasible for small size peptides (e.g. less than 6 amino acids). Since the molecules previously developed by two of the partners are hexa, deca and undecapeptides it may be highly expensive to be produced by standard chemical methods (e.g. 1000-3000 €/g).

Expression of AMPs in microbial systems such as in *E. coli* or *Pichia pastoris* have been reported (*124*, *125*). However, due to several constrains specific expression systems have to be developed to increase transgene size or to direct accumulation or secretion of the compounds. Apart from the moderate cost of production using large bioreactor facilities, microbial production of AMPs have the disadvantage of the high toxicity of the AMP to the producer microorganism, that can be solved with complicate strategies of expression that require protein refolding and protease processing after production. Finally the yield obtained in proteins similar to AMPs is usually low to moderate, in the range of 1 to 300 mg/l.

Numerous transgenic plants expressing natural AMPs have been developed in which self-protection against disease was observed (reviewed in 5, 4). Although there is a vast amount of AMPs expressed in plants with the objective of providing self-protection against fungal, bacterial or viral plant pathogens, rarely the approaches deal with the objective of producing high yields. The use of plants as biofactories offers interesting opportunities to produce proteins of great added value for pharmaceutical application (*126–129*). The advantages are the following: (a) the different types tissues from crop plants can be used as biofactories such as leaves (e.g. tobacco), seeds (e.g. rice, corn, soybean), tubers and fruits (e.g. potato) or fibre and oil crops (e.g. sunflower); (b) several strategies for expression of heterologous genes by using constitutive strong promoters or tissue specific-conditional promoters (c) different strategies to target the AMP to specific cell compartments or to express AMPs as fusion proteins linked through specific sequences for proteolytic cleavage; (d) the scalability, sustainability and safety as "green" production, greenhouse containment and self-pollinating crops; (e) the relatively low product recovery costs (depends on the strategy of expression and requirements)(< 85% of other systems like microbial or animal); and (f) the high yield reported for tissue and organelle specific accumulation strategies (up to 1% of total soluble protein, 1-10g/Kg of dry weight plant material).

Biotechnology companies produced or have ongoing projects to produce some antimicrobial peptides using plant biofactories based on barley, corn, tobacco, potato, rice and saponaria (*130*, *131*). Currently, an European consortium

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

is developing a project using rice, tobacco and potato biofactories to produce synthetic antifungal and antibacterial peptides.

4. Concluding Remarks

We have developed linear undecapeptides and cyclic decapeptides against plant pathogens following the classical hit-to-lead and lead optimization approaches, in these last case based on combinatorial chemistry of some critical positions in the sequence of the peptides.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

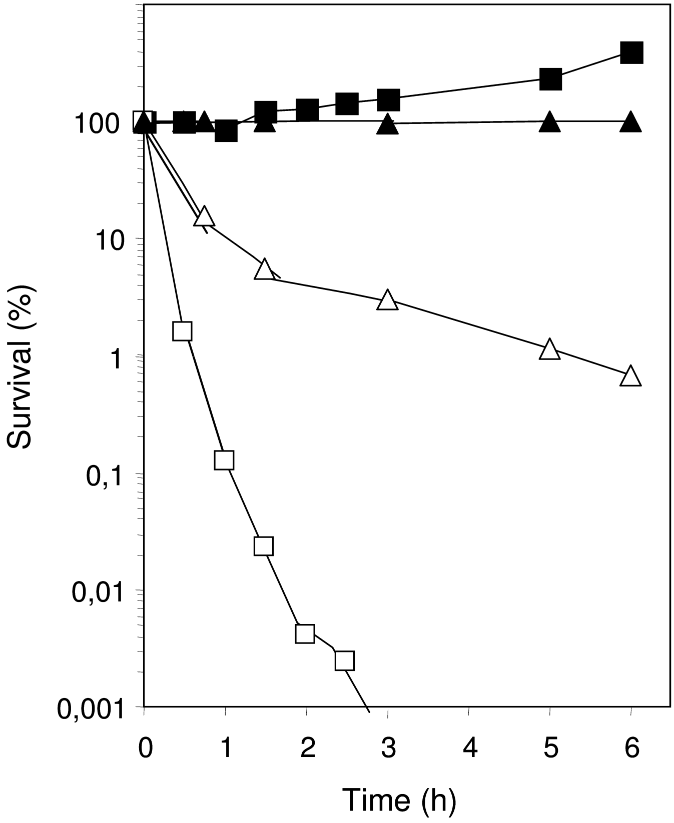


Figure 4. Kinetics of pathogen killing. Bactericidal activity of BP100 at 5 μM on E. amylovora (open squares) and sporicidal activity of BP21 at 25 μM on P.expansum (open triangles). Untreated controls of E. amylovora (filled squares) and P.expansum (filled triangles).

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

The linear undecapeptides of the CECMEL11 library composed of 125 members, departed from a peptide made of cecropin A-melittin chimeric domains that was improved for activity against plant pathogenic bacteria and fungi, but minimizing hemolytic activity and protease susceptibility. The selected peptides covered a wide range of action spectrum (Table 2). For example, peptide BP100 was strongly lytic against Gram negative bacteria including plant pathogenic bacteria and food-borne human pathogens (*Salmonella*, *E. coli*), but poorly antifungal. BP18 has a wide antibacterial spectrum including Gram positive (*Streptococcus* spp., *Listeria* spp.) and Gram negative bacteria, but it is also poorly antifungal. Contrarily, peptide BP21 displayed strong antifungal activity but slight antibacterial activity. BP15 was both antifungal and antibacterial, but in this last case only active against Gram negative bacteria. Within the library members there was in addition a second level of specificity in terms of the target microorganism. For example, BP15 was more active against *Fusarium oxysporum*, *P. syringae* than to *Penicillium expansum*, *E. amylovora* or *X. vesicatoria*. Interestingly, acute oral toxicity in mice for the three peptides tested (BP100, BP21 and BP15) is in the range of the slight to non-toxic compounds (higher than 1000 mg/g of body weight).

The cyclic decapeptides of the CYC10 library composed of 72 members, were designed *de novo* and were improved only against plant pathogenic bacteria, and for minimization of hemolytic activity. For example, peptide BPC92, c(Leu-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln) , was inactive against *E. amylovora* but highly active against *X. vesicatoria* and *P. syringae*.

The results with the two peptide libraries illustrate how subtle changes in a peptide sequence strongly influence antimicrobial activity, as well as other accompanying properties (e.g. toxicity, protease susceptibility), and that the specificity observed depend on both the peptide and the target microorganism.

The best peptides from the CECMEL11 library have bactericidal (BP100) or fungicidal (BP21) at the MIC concentrations with a potent effect. This is clearly shown in Figure 4 where BP100 decreased 3-log survival and BP21 1-log survival in an hour.

Because the main limitation of the implementation of the antimicrobial peptide technology in the field of plant protection is the production stage (technology and costs), strong efforts for an economically feasible and sustainable production are in progress. Great expectations are focused on the use of microbial and plant biofactories.

Acknowledgments

We acknowledge the Ministry of Science and Innovation-MICINN of Spain for grants AGL2009-13255-C02-AGR, PPT-060000-2008-2, and EUI2008-035725. The LIPPSO and CIDSAV groups are recognized as 2009SGR182 and 2009SGR812 by the Catalonian Government.

References

1. Agrios, G. N. *Plant Pathology*, 5th ed.; Academic Press: San Diego, CA, 2005.
2. McManus, P. S.; Stockwell, V. O.; Sundin, G.; Jones, A. L. *Annu. Rev. Phytopathol.* **2002**, *40*, 443–465.
3. Vidaver, A. K. *Clin. Infect. Dis.* **2002**, *34*, 107–110.
4. Marcos, J. F.; Muñoz, A.; Pérez-Payá, E.; Misra, S.; López-García, B. *Annu. Rev. Phytopathol.* **2008**, *46*, 271–301.
5. Montesinos, E. *FEMS Microbiol. Lett.* **2007**, *270*, 1–11.
6. Ajesh, K.; Sreejith, K. *Peptides* **2009**, *30*, 999–1006.
7. Hancock, R. E. W.; Sahl, H. G. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.
8. Jenssen, H.; Hamill, P.; Hancock, R. E. W. *Clin. Microbiol. Rev.* **2006**, *19*, 491–511.
9. Keymanesh, K.; Soltani, S.; Sardari, S. *World J. Microbiol. Biotechnol.* **2009**, *25*, 933–944.
10. Yount, N. Y.; Yeaman, M. R. *Protein Pept. Lett.* **2005**, *12*, 49–67.
11. Montesinos, E.; Bardají, E. *Chem. Biodivers.* **2008**, *5*, 1225–1237.
12. Settanni, L.; Corsetti, A. *Int. J. Food Microbiol.* **2008**, *121*, 123–138.
13. Ng, T. B. *Peptides* **2004**, *25*, 1055–1073.
14. Degenkolb, T.; Berg, A.; Gams, W.; Schlegel, B.; Gräfe, U. *J. Pept. Sci.* **2003**, *9*, 666–678.
15. Bonmatin, J. M.; Laprevote, O.; Peypoux, F. *Comb. Chem. High Throughput Screening* **2003**, *6*, 541–556.
16. De Lucca, A. J.; Walsh, T. J. *Antimicrob. Agents Chemother.* **1999**, *43*, 1–11.
17. Raaijmakers, J. M.; de Bruijn, I.; de Kock, M. J. D. *Mol. Plant-Microbe Interact.* **2006**, *19*, 699–710.
18. Stein, T. *Mol. Microbiol.* **2005**, *56*, 845–857.
19. Brady, S. F.; Wright, S. A.; Lee, J. C.; Sutton, A. E.; Zumoff, C. H.; Wodzinski, R. S.; Beer, S. V.; Clardy, J. *J. Am. Chem. Soc.* **1999**, *121*, 11912–11913.
20. Copping, G. L.; Menn, J. L. *Pest. Manag. Sci.* **2000**, *56*, 651–676.
21. Otvos, L., Jr. *J. Pept. Sci.* **2000**, *6*, 497–511.
22. Zasloff, M. *Nature* **2002**, *415*, 389–395.
23. Alan, A. R.; Earle, E. *Mol. Plant-Microbe Interact.* **2002**, *15*, 701–708.
24. Ferre, R.; Badosa, E.; Feliu, L.; Planas, M.; Montesinos, E.; Bardaji, E. *Appl. Environ. Microbiol.* **2006**, *72*, 3302–3308.
25. Kuzina, L. V.; Miller, T. A.; Cooksey, D. A. *Lett. Appl. Microbiol.* **2006**, *42*, 514.
26. López-García, B.; Pérez-Payá, E.; Marcos, J. F. *Appl. Environ. Microbiol.* **2002**, *68*, 2453–2460.
27. Cuthbertson, B. J.; Bullesbach, E. E.; Fievet, J.; Bachere, E.; Gross, P. S. *Biochem. J.* **2004**, *381*, 79–86.
28. Banzet, N.; Latorse, M. P.; Bulet, P.; François, E.; Derpierre, C.; Dubald, M. *Plant Sci.* **2002**, *162*, 995–1006.
29. Broekaert, W. F.; Cammue, B. P. A.; DeBolle, M. F. C.; Thevissen, K.; De Samblanx, G. W.; Osborn, R. W. *Crit. Rev. Plant Sci.* **1997**, *16*, 297–323.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

30. Castro, M. S.; Fontes, W. *Prot. Pep. Lett.* **2005**, *12*, 11–16.
31. Vila-Perelló, M.; Sánchez-Vallet, A.; García-Olmedo, F.; Molina, A.; Andreu, D. *FEBS Lett.* **2003**, *536*, 215–219.
32. Bohlmann, H.; Clausen, S.; Behnke, S.; Giese, H.; Hiller, C.; Reimann-Philipp, G. U.; Schrader; Barkholt, V.; Apel, K. *EMBO J.* **1988**, *7*, 1559–1565.
33. Terras, F. R. G.; Eggermont, K.; Kovaleva, V.; Raikhel, N. V.; Osborn, R. W.; Kester, A.; Rees, S. B.; Torrekens, S.; Van Leuven, F.; Vanderieyden, J.; Cammue, B. P. A.; Broekaert, W. F. *Plant Cell* **1995**, *7*, 573–588.
34. Schaaper, W. M.; Posthuma, G. A.; Plasman, H. H.; Sijtsma, L.; Fant, F.; Borremans, F. A.; Thevissen, K.; Broekaert, W. F.; Meloen, R. H.; van Amerongen, A. *J. Pept. Res.* **2001**, *57*, 409–418.
35. Andreu, D.; Rivas, L. *Biopolymers* **1998**, *47*, 415–433.
36. Brogden, K. A.; Ackermann, M.; McCray, P. B., Jr.; Tack, B. F. *Int. J. Antimicrob. Agents* **2003**, *22*, 465–478.
37. Bulet, P.; Stöcklin, R.; Menin, L. *Immunol. Rev.* **2004**, *198*, 169–184.
38. Ganz, T.; Lehrer, R. I. *Curr. Opin. Immunol.* **1998**, *10*, 41–44.
39. García-Olmedo, F.; Molina, A.; Alamillo, J. M.; Rodríguez-Palenzuela, P. *Biopolymers* **1998**, *47*, 479–491.
40. Giangaspero, A.; Sandri, L.; Tossi, A. *Eur. J. Biochem.* **2001**, *268*, 5589–5600.
41. Jenssen, H. *Expert. Opin. Drug. Discovery* **2011**, *6*, 171–184.
42. Papo, N.; Oren, Z.; Pag, U.; Sahl, H.-G.; Shai, Y. *J. Biol. Chem.* **2002**, *277*, 33913–33921.
43. Sato, H.; Feix, J. B. *Biochim. Biophys. Acta* **2006**, *1758*, 1245–1256.
44. Andreu, D.; Bruce, M. *Biopolymers* **2008**, *90*, 236–239.
45. Tossi, A.; Sandri, L.; Giangaspero, A. *Biopolymers* **2000**, *55*, 4–30.
46. Wiradharma, N.; Khoe, Y.; Hauser, C. A. E.; Seow, S. V.; Zhang, S.; Yang, Y-Y. *Biomaterials* **2011**, *32*, 2204–2212.
47. Zelezetsky, I.; Tossi, A. *Biochim. Biophys. Acta* **2006**, *1758*, 1436–49.
48. Blondelle, S. E.; Houghten, R. A. *Biochemistry* **1992**, *31*, 12688–12694.
49. Epand, R. F.; Lehrer, R. I.; Waring, A.; Wang, W.; Maget-Dana, R.; Lelièvre, D.; Epand, R. M. *Biopolymers* **2003**, *71*, 2–16.
50. Lee, S-H.; Kim, S-J.; Lee, Y-S.; Song, M-D.; Kim, I-H.; Won, H-S. *Regul. Pept.* **2011**, *166*, 36–41.
51. Oren, Z.; Hong, J.; Shai, Y. *J. Biol. Chem.* **1997**, *272*, 14643–14649.
52. Park, Y.; Leu, D. G.; Hahm, K. S. *Biotechnol. Lett.* **2003**, *25*, 1305–1310.
53. Monroc, S.; Badosa, E.; Feliu, L.; Planas, M.; Montesinos, E.; Bardají, E. *Peptides* **2006**, *27*, 2567–2574.
54. Monroc, S.; Badosa, E.; Besalú, E.; Planas, M.; Bardají, E.; Montesinos, E.; Feliu, L. *Peptides* **2006**, *27*, 2575–2584.
55. Blondelle, S. E.; Lohner, K. *Curr. Pharm. Des.* **2010**, *16*, 3204–3211.
56. Roth, B. L.; Poot, M.; Yue, S. T.; Millard, P. J. *Appl. Environ. Microbiol.* **1997**, *63*, 2421–2431.
57. Kaplan, C. W.; Sim, J. H.; Shah, K. R.; Kolesnikova-Kaplan, A.; Shi, W.; Eckert, R. *Antimicrob Agents Chemother.* **2011**, *55*, 3446–3452.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

58. Rathinakumar, R.; Walkenhorst, W. F.; Wimley, W. C. *J. Amer. Chem. Soc.* **2009**, *131*, 7609–7617.
59. Badosa, E.; Ferre, R.; Francés, J.; Bardají, E.; Feliu, L.; Planas, M.; Montesinos, E. *Appl. Environ. Microbiol.* **2009**, *75*, 5563–5569.
60. Reyes, R.; Ramírez, G.; Delgado, N. M. *Syst. Biol. Reprod. Med.* **2004**, *50*, 327–332.
61. De La Fuente-Salcido, N. M.; Salcedo-Hernández, R.; Alanis-Guzmán, G.; Bideshi, D. K.; Barboza-Corona, J. E. *J. Microbiol. Meth.* **2007**, *70*, 196–199.
62. Hadjicharalambous, C.; Sheynis, T.; Jelinek, R.; Shanahan, M. T.; Ouellette, A. J.; Gizeli, E. *Biochemistry* **2008**, *47*, 12626, 12634.
63. Friedrich, C. L.; Moyles, D.; Beveridge, T. J.; Hancock, R. E. W. *Antimicrob. Agents Chemother.* **2000**, *44*, 2086–2092.
64. Wu, M.; Maier, E.; Benz, R.; Hancock, E. W. *Biochemistry* **1999**, *38*, 7235–7242.
65. Epand, R. M.; Vogel, H. J. *Biochim. Biophys. Acta* **1999**, *1462*, 11–28.
66. Boman, H. G. *J. Intern. Med.* **2003**, *254*, 197–215.
67. Tenhami, M.; Hakkila, K.; Karp, M. *Antimicrob. Agents Chemother.* **2001**, *45*, 3456–3461.
68. Scott, M. G.; Yan, H.; Hancock, R. E. W. *Infect. Immun.* **1999**, *67*, 2005–2009.
69. Marchand, C.; Krajewski, K.; Lee, H. F.; Antony, S.; Johnson, A. A.; Amin, R.; Roller, P.; Kvaratskhelia, M.; Pommier, Y. *Nucleic Acids Res.* **2006**, *34*, 5157–5165.
70. Oita, S.; Ohnishi-Kameyama, M.; Nagata, T. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 958–964.
71. Brotman, Y.; Makovitzki, A.; Shai, Y.; Chet, I.; Viterbo, A . *Appl. Environ. Microbiol.* **2009**, *75*, 5373–5379.
72. Felix, G.; Regenass, M.; Boller, T. *Plant J.* **1993**, *4*, 307–316.
73. Ongena, M.; Jacques, P.; Toure, Y.; Destain, J.; Jabrane, A.; Thonart, P. *Appl. Microbiol. Biotechnol.* **2005**, *69*, 29–38.
74. Herman, M. A. B.; Davidson, J. K.; Smart, C. D. *Phytopathology* **2008**, *98*, 1226–1232.
75. Miyashita, M.; Oda, M.; Ono, Y.; Komoda, E.; Miyagawa, H. *ChemBioChem* **2011**, *12*, 1323–1329.
76. Scheer, J. M.; Pearce, G.; Ryan, C. A. *Planta* **2005**, *221*, 667–674.
77. Miyata, K.; Miyashita, M.; Nose, R.; Otake, Y.; Miyagawa, H. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 2138–2144.
78. Santos, J. A.; Gonzalez, C. J.; Otero, A.; Lopez, M. L. G. *Appl. Environ. Microbiol.* **1999**, *65*, 5612–5614.
79. Blondelle, S. E.; Lohner, K. *Biopolymers* **2000**, *55*, 74–87.
80. Ngamwongsatit, P.; Banada, P. P.; Panbangred, W.; Bunia, A. K. *J. Microbiol. Meth.* **2008**, *73*, 211–215.
81. Sharma, R. K.; Sundriyal, S.; Wangoo, N.; Tegge, W.; Jain, R. *ChemMedChem* **2010**, *5*, 86–95.
82. Wei, G-X.; Bobek, L. A. *Antimicrob. Agents Chemother.* **2005**, *49*, 2336–2342.

83. Errakhi, R.; Maimoun, P.; Lehner, A.; Vidal, G.; Briand, J.; Corbineau, F.; Rona, JP.; Bouteau, F. *J. Exp. Bot.* **2008**, *11*, 3121–3129.
84. Powell, W. A.; Catranis, C. M.; Maynard, C. A. *Mol. Plant-Microbe Interact.* **1995**, *8*, 792–794.
85. Makovitzi, A.; Viterbo, A.; Brotman, Y.; Chet, I.; Shai, Y. *Appl. Environ. Microbiol.* **2007**, *73*, 6629–6636.
86. Bessalle, R.; Kapitkovsky, A.; Gorea, A.; Shalit, I.; Fridkin, M. *FEBS Lett.* **1990**, *274*, 151–155.
87. Oh, D.; Shin, S. Y.; Lee, S.; Kang, J. H.; Kim, S. D.; Ryu, P. D.; Hahm, K. S.; Kim, Y. *Biochemistry* **2000**, *39*, 11855–11864.
88. Juretic, D.; Chen, H.; Brown, J.; Morell, J. L.; Hendler, R. W.; Westerhoff, H. V. *FEBS Lett.* **1989**, *249*, 219–223.
89. Nishi, N.; Itoh, A.; Fujiyama, A.; Yoshida, N.; Araya, S.; Hiroshima, M.; Shoji, H.; Nakamura, T. *FEBS Lett.* **2005**, *579*, 2058–2064.
90. Shibata, K.; Suzawa, T.; Soga, S.; Mizukami, T.; Yamada, K.; Hanai, N.; Yamasaki, M. *Bioorg. Med. Chem.* **2003**, *13*, 2583–2586.
91. Cudic, M.; Lockatell, C. V.; Johson, D. E.; Otvos, L. *Peptides* **2003**, *24*, 807–820.
92. Güell, I.; Cabrefiga, J.; Badosa, E.; Ferre, R.; Talleda, M.; Bardají, E.; Planas, M.; Feliu, L.; Montesinos, E. *Appl. Environ. Microbiol.* **2011**, *77*, 2667–2675.
93. Wilson-Stanford, S.; Kalli, A.; Hakansson, K.; Kastrantas, J.; Orugunty, R. S.; Smith, L. *Appl. Environ. Microbiol.* **2009**, *75*, 1381–1387.
94. Dashper, S. G.; O'Brien-Simpson, N. M.; Cross, K. J.; Paolini, R. A.; Hoffmann, B.; Catmull, D. V.; Malkoski, M.; Reynolds, E. C. *Antimicrob Agents Chemother.* **2005**, *49*, 2322–2328.
95. Ali, G. S.; Reddy, A. S. N. *Mol. Plant-Microbe Interact.* **2000**, *13*, 847–859.
96. Cavallarin, L.; Andreu, D.; San Segundo, B. *Mol. Plant-Microbe Interact.* **1998**, *11*, 218–227.
97. Matsuzaki, K.; Sugishita, K.; Fujii, N.; Miyajima, K. *Biochemistry* **1995**, *34*, 3421–3429.
98. Badosa, E.; Ferre, R.; Planas, M.; Feliu, L.; Besalú, E.; Cabrefiga, J.; Bardají, E.; Montesinos, E. *Peptides* **2007**, *28*, 2276–2285.
99. Matsuzaki, K. *Biochim. Biophys. Acta* **2009**, *1788*, 1687–1692.
100. Hong, S. Y.; Oh, J. E.; Lee, K-H. *Biochem. Pharmacol.* **1999**, *58*, 1775–1780.
101. Li, X.; Li, Y.; Han, H.; Miller, D. W.; Wang, G. *J. Am. Chem. Soc.* **2006**, *128*, 5776–5785.
102. Wessolowski, A. M.; Bienert, M.; Dathe, M. *J. Peptide. Res.* **2004**, *64*, 159–169.
103. Zhu, W. L.; Nan, Y. H.; Hahm, K.-S.; Shin, S. Y. *J. Biochem. Mol. Biol.* **2007**, *40*, 1090–1094.
104. Dathe, M.; Nikolenko, H.; Klose, G.; Bienert, M. *Biochemistry* **2004**, *43*, 9140–9150.
105. Frecer, V.; Ho, B.; Ding, J. L. *Antimicrob. Agents Chemother.* **2004**, *48*, 3349–3357.
106. Muhle, S. A.; Tam, J. P. *Biochemistry* **2001**, *40*, 5777–5785.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

107. Reed, J. D.; Edwards, D. L.; Gonzalez, C. F. *Mol. Plant-Microbe Interact.* **1997**, *10*, 537–549.

108. Muñoz, A.; López-García, B.; Marcos, J. F. *Antimicrob. Agents Chemother.* **2006**, *50*, 3847–3855.

109. Brogden, K. A. *Nat. Rev. Microbiol.* **2005**, *3*, 238–250.

110. Yeaman, M. R.; Yount, N. Y. *Pharmacol. Rev.* **2003**, *55*, 27–55.

111. Beckinger, B. *Crit. Rev. Plant. Sci.* **2004**, *23*, 271–292.

112. Beckinger, B.; Lohner, K. *Biochim. Biophys. Acta* **2006**, *1758*, 1529–1539.

113. Kalfa, V. C.; Jia, H. P.; Kunkle, R. A.; McCray, P. B., Jr.; Tack, B. F.; Brogden, K. A. *Antimicrob. Agents Chemother.* **2001**, *45*, 3256–3261.

114. Lohner, K.; Blondelle, S. E. *Comb. Chem. High Throughput Screen* **2005**, *8*, 241–256.

115. Hirakura, Y.; Alvarez-Bravo, J.; Kurata, S.; Natori, S.; Kirino, Y. *J. Biochem.* **1996**, *120*, 1130–1140.

116. Tran, H.; Ficke, A.; Asiimwe, T.; Höfte, M.; Raaijmakers, J. M. *New Phytol.* **2007**, *175*, 731–742.

117. Deshayes, S.; Morris, M. C.; Divita, G.; Heitz, F. *Cell. Mol. Life Sci.* **2005**, *62*, 1839–1849.

118. Eggenberger, K.; Mink, C.; Wadhwani, P.; Ulrich, A. S.; Nick, P. *ChemBioChem* **2011**, *12*, 132–137.

119. Ferre, R.; Melo, M. N.; Correia, A. D.; Feliu, L.; Bardají, E.; Planas, M.; Castanho, M. *Biophys. J.* **2009**, *96*, 1815–1827.

120. Alves, C. S.; Melo, M. N.; Franquelim, H. G.; Ferre, R.; Planas, M.; Feliu, L.; Bardají, E.; Kowalczyk, W.; Andreu, D.; Santos, N. C.; Fernandes, M. X.; Castanho, M. *J. Biol. Chem.* **2010**, *285*, 27536–27544.

121. Mika, J. T.; Moiset, G.; Cirac, A. D.; Feliu, L.; Bardají, E.; Planas, M.; Sengupta, D.; Marrink, S. J.; Poolman, B. *Biochim. Biophys. Acta* **2011**, *1808*, 2197–205.

122. Kunkel, B. N.; Brooks, D. M. *Current Opin. Plant Biol.* **2002**, *5*, 325–332.

123. Durrant, W. E.; Dong, X. *Annu. Rev. Phytopathol.* **2004**, *42*, 185–209.

124. Graumann, K.; Premstaller, A *Biotechnol. J.* **2006**, *1*, 164–186.

125. Morin, K. M.; Arcidiacono, S.; Beckwitt, R.; Mello, C. M. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 698–704.

126. Twyman, R. M.; Stoger, E.; Schillberg, S.; Christou, P.; Fischer, R. *Trends Biotechnol.* **2003**, *21*, 570–578.

127. Fischer, R.; et al. *Curr. Opin. Plant Biol.* **2004**, *7*, 152–158.

128. Teli, N. P.; Timko, M. P. *Plant Cell, Tissue Organ Cult.* **2004**, *79*, 125–145.

129. Biemelt, S.; Sonnewald, U.; Galmbacher, P.; Willmitzer, L.; Müller, M. *J. Virol.* **2003**, *77*, 9211–9220.

130. Nandi, S.; Yalda, D.; Lu, S.; Nikolov, Z.; Misaki, R.; Fujiyama, K.; Huang, N. *Transgenic Res.* **2005**, *14*, 237–240.

131. Spök, A. *Trends Biotechnol.* **2006**, *25*, 74–82.

Downloaded by UNIV OF GUELPH LIBRARY on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch012

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Chapter 13

Antimicrobial Peptides as a Promising Alternative for Plant Disease Protection

B. López-García, B. San Segundo, and M. Coca*

**CRAG-Center for Research in Agricultural Genomics
(CSIC-IRTA-UAB-UB), Edificio CRAG, Campus de la UAB,
08193 Bellaterra, Barcelona, Spain
*E-mail: maria.coca@cragenomica.es**

Plants produce antimicrobial peptides (AMPs) to defend themselves against pathogens. The repertoire of AMPs synthesized by plants is extremely large, with hundreds of different AMPs in some plant species. In spite of their molecular diversity most plant AMPs share common features: they are basic, amphypatic and cysteine-rich peptides with a stabilized structure by disulfide bonds. Plant AMPs antimicrobial activity is not only against plant pathogens and predatory insects, but also against human viruses, bacteria, fungi, protozoa parasites and neoplastic cells. Thus, plant AMPs are considered as promising antibiotic compounds with important biotechnological applications. This review describes the different plant AMP classes and their natural functions in plant defense. It also discusses the biotechnological applicaticons of AMPs, either natural or synthetic, in plant disease protection. Finally, the use of plants as biofactories is presented as an alternative for the production of AMPs.

Introduction

Plants are constantly exposed to a variety of microorganisms, yet they are resistant to the vast majority of potential pathogens. Plants have evolved a surveillance system that detects invading pathogens and induces resistance mechanisms to control pathogen attack. The initiation of defense responses depends on the recognition of pathogen epitopes, also known as

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

pathogen-associated molecular patterns (PAMPs), by receptors at the plant's cell surface, a phenomenon often referred as basal disease resistance (*1*). The perception of PAMPs activates a complex process in which different signalling cascades operate leading to the transcriptional activation of defense-related genes. Successful pathogens are also known to produce effectors to inhibit PAMP-triggered immunity, but plants, in turn, can perceive such effectors through additional receptors to mount another layer of defense called effector-triggered immunity (formerly known as gene-for-gene resistance) (*2*, *3*). The plant response to pathogen attack includes the rapid generation of reactive oxygen species, the reinforcement of cell wall, as well as the production of small molecules (phytoalexins), pathogenesis related (PR) proteins, and other antimicrobial peptides (AMPs) (*4*, *5*). Resistance responses locally activated in primary pathogen-infected plant tissues are often extended to distant, non-infected tissues, conferring an elevated level of protection. This phenomenon referred as systemic acquired resistance is correlated with the induction of PR genes and is long lasting effective against a broad spectrum of pathogens (*5*).

Different plant peptides that inhibit the growth of microorganism have been identified as AMPs (*6*). The general features of plant AMPs are small molecular size, net positive charge, amphipathic properties, and rich in cysteine residues conferring a high termostability. Recent analyses suggest that plant genomes are rich in genes encoding cysteine-rich peptides resembling AMPs, which might account for up to 2-3% of the predicted genes, suggesting that plant possess a formidable defense arsenal (*7*). In addition to AMPs, the so-called PR proteins exhibit antimicrobial activity and their accumulation in the plant correlates with pathogen resistance. They have been classified into 17 families, some of them consist of proteins with a molecular size below 10 kDa, called pathogenesis-related peptides (*4*). These PR peptides include proteinase inhibitors (PR-6 family), plant defensins (PR-12 family), thionins (PR-13 family) and lipid transfer proteins (PR-14 family), and share the general features of AMPs. In fact, these families of PR peptides are included into the broad class of plant AMPs.

In addition to plants, peptides and small proteins with antimicrobial activity have been characterized from most living organisms ranging from insects to humans and have prevailed throughout evolution as components of the innate defense against microbial invasion (*8*). Many of these AMPs exhibit a broad spectrum of antimicrobial activity *in vitro*, inhibiting the growth of Gram-positive and Gram-negative bacteria, fungi, some protozoan parasites and viruses. The fast, efficient and durable action of these 'natural antibiotics' against microbes made them potential candidates as peptide drugs and several examples are undergoing clinical trials in biomedicine (*9*). The potential application of AMPs has also been extended to plant protection and as biopreservatives in the food industry (*10–12*).

In this book chapter, we will describe the different classes of plant AMPs, their function as innate defense components, and their biotechnological applications. The use of AMPs from different sources, natural and synthetic, in plant protection is also included in this chapter. A broad description of AMPs from sources other than plants is not intended and only the distinguished peptides, for which a role in

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

plant protection is well established, will be described. Finally, the use of plants as biofactories is presented as alternative AMP production system.

Overview of Antimicrobial Peptides and Proteins

AMPs are a broad class of peptides and small proteins of less than 100 amino acids (most of them less than 50 amino acids), and they can be sub-divided into several groups based on their origin, composition and structure (*6*, *13*). The vast amount of data on natural AMPs has propelled the development of several databases, such as the Collection of Anti-Microbial Peptides *CAMP* (www.bicnirrh.res.in/antimicrobial) (*14*), the Plant AMP Database *PhytAMP* (phytamp.pfba-lab-tun.org) (*15*), and the Antimicrobial Peptide Database *ADP* (aps.unmc.edu/AP/main.html) (*16*).

In general, most AMPs are positively charged at physiological pH due to an excess of basic residues such as arginine and lysine, although some anionic AMPs have also been reported (*17*). In addition, they contain hydrophobic residues such as alanine, leucine, phenylalanine and tryptophan. From a structural point of view, AMPs are peptides that fold into different conformations, including α-helices, β-sheets, extended and looped. Many of them adopt an amphipathic structure under specific experimental conditions, a feature that determines their mode of action on microorganisms.

Some AMPs are rich in certain amino acids (*13*). This is the case of proline-rich peptides such as apidaecins from honeybees; bactenecins from cattle, sheep, and goats; and PR-39 from pigs. Also, some peptides are relatively rich in glycine such as hymenoptaecin from honeybees and shepherin I and II from shepherd's purse plants. Moreover, there are some AMPs with an unusually high content of tryptophan (i.e. indolicidin from cattle) or histidine (i.e. histatins from human and some higher primates). A highly abundant class is cysteine-rich peptides with disulfide bonds that make these peptide structures compact and remarkably stable to adverse biochemical conditions and protease degradation. Animal and insect defensins, antifungal proteins from fungi, and most of the antimicrobial peptides found in plants belong to this class (*13*, *18*).

Several works have reviewed the mode of action of different AMPs (*11*, *19*, *20*). Concerning cationic AMPs, different studies conclude that the primary step is the electrostatic interaction between the peptide and the negative-charged microbial membranes. AMPs interact with specific phospholipid domains or lipid rafts (*21*). Based on their amphipathic properties, AMPs are able to insert into, and disrupt, lipid bilayers. For many AMPs, the destabilization of lipid membranes is correlated with permeation and antimicrobial activity (*22*, *23*). However, this is not the primary mode of action of all AMPs and other subtle mechanisms may be associated to their antimicrobial activity (*13*, *20*). This idea is supported by the identification of some AMPs that are able to cross biological membranes without cell permeation (*24*, *25*). Once inside the cell, these cell-penetrating antimicrobial peptides may alter different intracellular processes by binding to DNA, RNA or proteins that lead to cell death and subsequent cell permeation. For some AMPs, both modes of action have been demonstrated; they induce membrane permeation and they are also able to internalize into the cell and bind nucleic acids (*25*, *26*). The balance between cell-penetration and cell-permeation may depend on the concentration of AMPs.

Plant Antimicrobial Peptides and Proteins

Different AMP families have been identified and characterized in plants (*reviewed by* (*4*, *6*)), and *in vitro* antimicrobial properties have been demonstrated for all of them. Some years ago, a database specifically designed for plant AMPs was created (PhytAMP, phytamp.pfba-lab-tun.org), which contains their microbiological, physicochemical and structural properties (*15*). One specific characteristic of plant AMPs is that most of these peptides are rich in cysteine forming disulfide bonds. Other amino acids abundant in these peptides are glycine followed by proline.

Most plant AMPs are processed from a precursor which consist of an N-terminal signal peptide and the mature AMP. In some cases, it has been demonstrated that the amino-signal peptide targets the AMP to the cell secretory pathway where they are exported to the apoplast. In addition, most AMP precursors have an acidic peptide in C-terminal (e.g. thionins and some floral defensins) or in N-terminal (e.g. snakins) of the mature AMP that serve to neutralize the basic AMP.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Thionins

Currently, the thionins family includes α1- and β-purothionins, α- and β-hordothionins, phoratoxin-A, *Pyrularia pubera* toxin and viscotoxin A1, A3 and B2. They have been identified in different organs (leaves, stems, seeds and roots) of a wide range of monocotyledonous and dicotyledonous plant species where they are encoded by genes displaying organ-specific expression (*27*). Thionins represent a family of small cysteine-rich peptides (about 5 kDa) ranging from 45 to 48 amino acids in length and usually basic. The presence of three or four conserved disulfide bonds leads to a common compact fold called Γ-fold, characterized by the presence of two domains: the vertical stem consisting of a pair of antiparallel α-helices and the horizontal arm formed by a coil in extended conformations, β-turn and an antiparallel β-sheet (Figure 1A) (*18*).

Thionins have broad *in vitro* antimicrobial activity against several Gram-positive and Gram-negative plant pathogenic bacteria, as well as different phytopathogenic fungi with IC_{50} values (concentration required for 50% growth inhibition) ranging from 0,2 to 3 μM (*28*, *29*). Some Gram-negative bacteria such as a number of *Pseudomonas* and *Erwinia* species are, however, insensitive to thionins. It has been suggested that the covalent binding of thionins to one periplasmic component of the pathogenic bacterium *Pseudomonas solanacearum* could be related with resistance of the pathogen to the peptide (*30*). In addition to microorganisms, they are also toxic to insect and mammalian cells (*31*, *32*).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Plant Defensins

Defensins constitute the unique class of AMPs involved in the innate immune response that seems to be conserved between plants, invertebrates and vertebrates (*33*, *34*). The first plant defensins isolated from wheat and barley were termed γ-thionins, but based on their resemblance to the insect and mammalian defensins they were redefined as plant defensins (*35*). The plant defensin family is quite numerous and ubiquitous with members isolated from both monocotylodenous and dicotylodenous plants. They have been purified from different plant tissues such as seeds, stems, roots, leaves and flowers. Their preferential localization in the peripheral cell layers, stomatal cells or phloem area in leaves is consistent with a role in protection against microbial challenge (*36*, *37*). Plant defensins are small (45-55 amino acids) highly basic cysteine-rich peptides. Their 3D structure presents three stranded, anti-parallel β-sheets and one α-helix following a βαββ pattern with 8 cysteines forming four disulfide bridges that stabilize this characteristic α/ β structure (CSαβ motif) (Figure 1B).

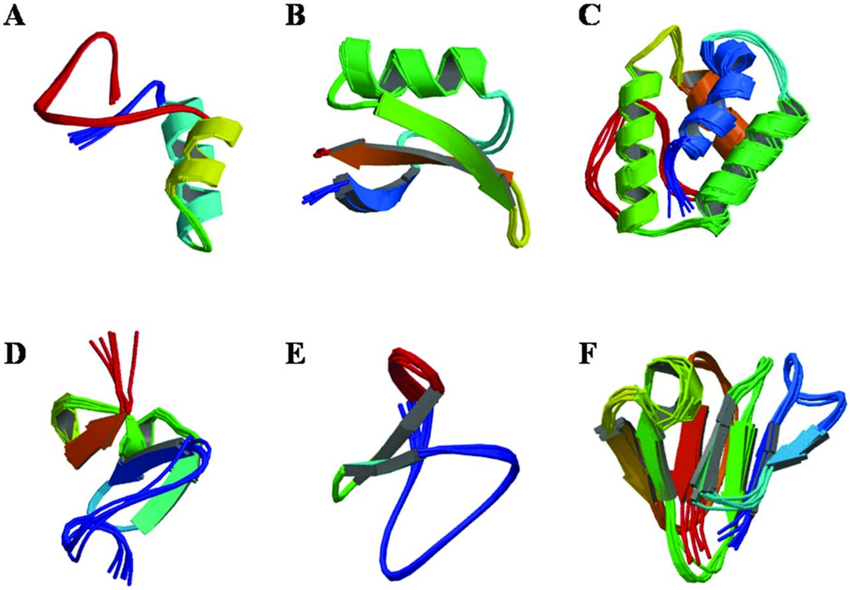


Figure 1. Representative tertiary structure for different plant AMP families. A) Thionins (viscotoxin-A3, PDB entry 1ED0), B) Defensins (Ah-AMP1, PDB entry1BK8), C) LTPs (Zm-LTP1, PDB entry 1AFH), D) Hevein (PDB entry 1HEV), E) Cyclotides (kalata B1, PDB entry 1JJZ), F) β-barrilin (MiAMP1, PDB entry 1C01).

Plant defensins have the ability to inhibit the *in vitro* growth of a broad range of filamentous fungi and yeast (*33*) and have been classified into two groups based on their effect on the fungal growth: morphogenic and non-morphogenic defensins. For some non-morphogenic defensins activity against several Gram-positive bacteria has been demonstrated, i.e., the *Br*-AFP2 identified from *Brassicaceae rapa* seeds has a lower antifungal activity but an enhanced antibacterial activity compared to *Rs*-AFP2 isolated from *Raphanus sativus* seeds (*38*).

Lipid Transfer Proteins (LTPs)

LTPs were firstly identified for their ability to transfer phospholipids between membranes *in vitro*. In consequence, plant LTPs were hypothesized to be involved in intracellular lipid trafficking, but nowadays there is no clear evidence of this biological function for LTPs.

Two LTP subfamilies were defined based on molecular mass: 9KDa LTP1 and 7KDa LTP2 (*39*). Although both groups have a high pI and share a similar 8 cysteine motif, they exhibit low amino acid sequence similarity and differ in their disulfide bond connectivities. LTP structure consists on a hydrophobic tunnel-like cavity formed by 4 helices stabilized by 4 disulfide bonds (Figure 1C). This cavity has been proposed as the lipid binding site. The higher flexibility of the cavity of LTP2 compared with LTP1 could explain why LTP2 transfer lipid molecules more efficiently than LTP1 (*39*, *40*). Recently, a new type of plant lipid transfer protein has been isolated from *Arabidopsis thaliana*. This protein called DIR1 shares some structural and lipid binding properties with plant LTP2, but displays some specific features such as an anionic character (pI of 4.25) that makes DIR1 unique in the LTP family (*41*).

LTP1 is found primarily in aerial organs, whereas LTP2 is expressed in roots. Interestingly, both classes are found in seeds. Several studies have revealed a localization of LTP1 at the cell wall of different plant species (*42*, *43*). Based on their extracellular localization, a role in intracellular lipid transport is considered unlikely for LTPs. In contrast, a LTP1 of *Triticum aestivum* seeds (LTP1e$_1$), that is not able to inhibit fungal growth, was specifically localized within aleurone cells, but not in the cell walls of mature wheat seeds (*44*).

The antibiotic properties of LTPs were discovered by screening plant proteins for their ability to inhibit the growth of several fungal and bacterial pathogens (*45*, *46*). The identification of an LTP-like protein in onion seeds, Ace-AMP1, with strong antimicrobial activity but without lipid-binding activity, supports that at least for Ace-AMP1 the observed antimicrobial activity is not related to a lipid-binding activity (*46*). Curiously, Ace-AMP1 is the most potent peptide belonging to the LTP family, showing inhibitory activity against fungi and Gram-positive bacteria at concentrations below of 1 μM.

Puroindolin A and B are two peptides structurally related to LTPs identified in wheat seeds. They contain five disulfide bridges and a tryptophan-rich domain. Puroindolins are able to inhibit the *in vitro* growth of some pyhtopathogenic fungi (e.g. *Botrytis cinerea, Verticillium dahliae, Fusarium culmorum,* and *Alternaria*

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

brassicola). The synthesis of ns-LTP1 and puroindolins in wheat are temporally and spatially regulated (*44*).

Snakins

The first members of this family were purified from potato tubers and called snakin-1 (StSN1, 63 amino acids) and snakin-2 (StSN2, 66 amino acids). Both antimicrobial peptides show similarity with members of the GAST (giberellic acid stimulated transcript) and GASA (giberellic acid stimulated in Arabidopsis) protein families from Arabidopsis. Currently, orthologues of snakins have been predicted in other plants, including maize (ZmGASA-like), rice (GAST1), and tomato (RSI-1) (*PhytAMP,* phytamp.pfba-lab-tun.org).

Snakin peptides are basic and contain 12 conserved cysteine residues which may form six disulfide bridges that stabilize their structure. Some motifs of snakin peptides share certain similarity with Cys-rich domains from animal proteins, such as hemotoxic snake venoms. StSN1 and StSN2 are active against bacterial and fungal pathogens at concentrations lower than 10 µM. Both snakins show almost identical antimicrobial activity spectra in spite of their low sequence similarity. The combined effect of StSN1 and the potato defensin StPTH1 was synergistic against the bacteria *Clavibacter michiganensis* subsp. *sepedonicus*, but additive against the fungus *B. cinerea* (*47*). Until now, the mode of action of snakins remains unknown. In contrast with other plant AMPs, they appear not to interact with artificial lipid membranes. Both snakins peptides cause a rapid aggregation of Gram-positive and Gram-negative bacteria *in vitro*, although this aggregation did not correlate with antimicrobial activity. However, aggregation could still play a role in the control of the pathogen *in vivo*. Recently, a new peptide CaSnakin was identified in pepper with high homology to StSN2 and strong activity against nematodes (*48*).

Genes encoding StSN1 and StSN2 are differentially expressed in plant tissues and in response to biotic and abiotic stress. The *StSN1* gene is constitutively expressed in plant tissues during development and does not respond to abiotic or biotic stress (*47*). In contrast, the *StSN2* expression gene is induced by wounding and fungal infection and repressed by bacterial infection (*49*).

Hevein-like Peptides

This family is formed by different small chitin-binding peptides that have a cysteine/glycine-rich domain homologue to that of other chitin-binding proteins isolated from plants, such as lectins and chitinases. The best known member is hevein, a 43-residue antifungal peptide isolated from rubber tree latex (*50*). Other peptides homologous to hevein but with higher antifungal potency have been isolated from different plants. In contrast to hevein, which is anionic (pI of 4.63), hevein-like peptides are small cationic peptides (29-45 residues, pI ≥ 8).

Considering the cysteine residues forming disulfide bridges, they can be classified in three groups. The first group comprises the hevein and other hevein-like peptides, such as Pn-AMPs isolated from *Pharbitis nil* seeds (*51*), and Fa-AMPs from *Fagopyrum esculentum* Moench (*52*), characterized by 8

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

cysteine residues. The second group includes some hevein-like peptides that have 6 cysteine residues, including *Ac*-AMPs identified from *Amaranthus caudatus* (*53*), IWF4 from *Beta vulgaris* (*54*), and Ar-AMP from *Amaranthus retroflexus* seeds (*55*). Finally, the third group comprises some peptides such as EAFPs purified from *Eucommia ulmoides* (*56*) and Ee-CBP from *Euonymus europaeus* (*57*) which are stabilized by 5 disulfide bonds.

The structure of hevein is characterized by a three-stranded β-sheet and a short single turn α-helix connecting the second to the third β-strands (Figure 1D). EAFP2, a typical hevein-like peptide with 5 disulfide bridges, adopts a compact global fold composed of a 3_{10} helix, an α-helix, and a three-strand antiparallel β-sheet. The most significant feature of EAFP2 is a well-defined amphipathic surface in contrast to the non-amphipathic topology of hevein (*58*).

Hevein-like peptides inhibit fungal growth at much higher extent than other previously characterized antifungal chitin-binding proteins. They show potent activity against different fungi and some Gram-positive bacteria, but they do not affect most Gram-negative bacteria. These peptides show remarkable stability to heat treatment, protease degradation or wide pH conditions. Some hevein-like peptides are active against chitin-containing and chitin-free fungi suggesting that chitin binding affinity may be not essential to exert a fungal inhibitory activity (*51*, *56*). There is little information about the expression of hevein-like genes and the localization of corresponding peptides. *IWF4* mRNA is expressed in the aerial parts of the beet plants only, with a constitutive expression in young and mature leaves and in young flowers. Its expression is not induced during infection (*54*). Other hevein-like peptides such as *Ac*-AMPs are present in seeds (*53*).

Knottin-like Family

Knottins are small disulfide-rich proteins characterized by a very special knot shaped when one disulfide bridge crosses the macrocycle formed by the two other disulfides and the interconnecting backbone. This knot is called 'disulfide through disulfide knot' motif and implies at least 3 disulfide bridges. Due to the exceptional stability of the knottin-like motif, it is a promising scaffold for drug development to pharmaceutical and agrochemical applications (*59*). The knottin structural family includes several unrelated families (http://knottin.cbs.cnrs.fr/) (*60*). Regarding to knottin-like plant AMPs we can distinguish different families of peptides of 30-40 amino acids, being the cyclotides the most widely studied.

Cyclotides

Cyclotides form a unique family of cyclic knotted peptides isolated from plants of the Violaceae, Rubiaceae, Cucurbitaceae and Fabaceae families (*reviewed by* (*61*)). Currently there are more than 300 sequences documented in the cyclic protein database called Cybase (www.cybase.org.au) (*62*).

Cyclotides are gene-encoding products derived from the processing of a large precursor protein containing an ER signal sequence, a Pro-region, a highly conserved N-terminal repeat region (NTR), the mature cyclotide domain, and a short C-terminal tail. Individual cyclotide genes encode between one and three repeats of the NTR and the cyclotide domain to form multiple cyclotides from a single precursor (*61*). Mature cyclotides are small head-to-tail cyclic peptides with typical masses of 2.5-4 kDa and six cysteine residues absolutely conserved in all of them. In contrast to most of the antimicrobial peptides identified in all the organisms, several cyclotides posses a negative net charge, e.g. kalata-B3, cyclotide Hyfl-B and cycloviolacin-O23. These peptides are characterized by the so called cyclic cysteine knot structural motif (CCK) which provides exceptional chemical and biological stability (Figure 1E) (*63*). The cyclic backbone of cyclotides is not essential for the *in vivo* formation of the CCK motif, as was shown by the characterization of the linear cyclotide violacin A that adopts the typical cyclotide fold despite having a non-cyclic backbone (*64*).

The inhibitory activity of bacterial and fungal growth has been confirmed for several members of the cyclotide family, e.g. kalata, circulin A and cycloviolacin O2 (*61*). However, there is no record of activity of this peptide family against phytopathogens. Craik and coworkers demonstrated that the roots and the aerial counterparts of various Viola species contain a large number of different cyclotides (*65*). They found clear variations in the cyclotide profiles of different parts of the plant showing a tissue-specific expression. All plant parts in contact with soil produce more hydrophobic cyclotides.

Other Knottin-like Peptides

The first plant knottin-like peptides were isolated from *Mirabilis jalapa* seeds and called *Mj*-AMP1 and *Mj*-AMP2 (*32*). These peptides contain 6 cysteine residues. They exhibit a broad spectrum of antimicrobial activity against phytopathogenic fungi (e.g. *B. cinerea, Colletotrichum lindemathianum* and *Venturia inaequalis*) and Gram-positive bacteria (e.g. *Bacillus megaterium*). *Mj*-AMPs are not toxic to Gram-negative bacteria (e.g. *Erwinia carotovora*), and human cells.

Afterwards, more peptides with a 'disulfide through disulfide knot' motif have been identified from different plant species, such as PAFP-s isolated from *Phytolacca americana* seeds (*66*) and 6-cysteine knottin-like peptides isolated from wheat seeds (*67*). PAFP-s have antifungal activity against *Fusarium oxysporum, Fusarium graminearum, Alternaria tennuis* and *Magnaporthe oryzae*. Floral defensins containing 5 disulfide bridges and isolated from *Petunia hybrid,* PhD1 and PhD2 (*68*), have been also classified as knottin-like peptides in the knottin database (http://knottin.cbs.cnrs.fr/).

Moreover, enzyme inhibitors with the knottin scaffold have been isolated from plants, including proteinase (carboxypeptidase and serine protease) and α-amylase inhibitors. Even though proteinase inhibitors have been traditionally associated to the plant defense against insect attack, evidence exists for a role in resistance to fungal pathogens (*69*).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

β-Barrelins

Mi-AMP1 is a cationic peptide isolated from *Macadamia integrifolia* with 76 amino acids, including 6 cysteine residues without significant sequence similarity to previously described peptides (*70*). It is a potent antimicrobial peptide active against fungal phytopathogens, oomycete phytopathogens and the Gram-positive bacteria *Clavibacter michiganensis*. MiAMP was non-toxic against Gram-negative bacteria, three human mycopathogens, plant and mammalian cells. The tridimensional structure consist of eight β-strands which are arranged in two Greek key motifs that form a Greek key β-barrel (Figure 1F) (*71*). This structure, called β-barrelin, is unique in plant AMPs and shows similarity to the yeast killer toxin WmKT, an inhibitor of β-glucan synthesis.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

2S Albumins

2S albumins are a family of small storage proteins rich in glutamine and cysteine residues isolated from monocotyledonous and dicotyledonous seeds. They are formed by two subunits; the large subunit of 8-14 kDa and the small one of 3-10 kDa. Structurally, 2S albumins are characterized by 5 amphipathic helices folded in a right-handed superhelix with 4 cysteine-bridges, a folding motif related to LTP (*72*). Different works have shown their *in vitro* antimicrobial activity against fungal phytopathogens, including *F. oxysporum, Fusarium solani* and *Colletotrichum* spp, and against some human pathogenic bacteria and yeast (*73*).

Four-Cysteine Antimicrobial Peptides

There are three families of peptides with 4 cysteine residues currently reported: the MBP-1 peptide purified from *Zea mays* L. seeds and homologues (*74*), the group of 4 peptides isolated from *Impatiens balsamina* seeds (i.e., Ib-AMP1, Ib-AMP2, Ib-AMP3 and Ib-AMP4) (*75*), and the MiAMP2 family purified from *Macadamia integrifolia* seeds (*76*).

MBP-1 is a cationic α-helical peptide 33 residues long that inhibits the *in vitro* growth of both bacteria and fungi (*74*). Based on the cystein motif and number, three AMPs from wheat Tk-AMP-X1, -X2, and -X3 were identified as MBP-1 homologues (*67*).

All four Ib-AMPs are 20 amino acid long, being the smallest AMPs found so far in plants. They are encoded within a single transcript and the Ib-AMP precursor protein consists of a prepeptide followed by 6 mature peptide domains, each flanked by acidic propeptide domains ranging from 16 to 34 amino acids in length (*75*). These peptides showed potent inhibitory activity against a range of filamentous fungi, yeast, and Gram-positive bacteria, but they are not cytotoxic to Gram-negative bacteria and cultured human cells. The mode of action of these small peptides is not clear. It has been demonstrated that Ib-AMP3 is able to bind strongly chitin, mannan and sphingomyelin, and weakly to galactocerebrosides, β-1,3-glucan, ergosterol and cholesterol (*77*).

Another example of AMPs processed from a unique multipeptide precursor is MiAMP2 containing several members (MiAMP2a, b, c, and d) which are processed by cleavage of the proximal N-terminal hydrophilic cysteine-rich sequence of vicilin from Macadamia (*76*). They constitute the vicilin-like AMP family. Two members, the MiAMP2a and the MiAMP2b, constitute some of the few anionic antimicrobial peptides so far described. Plant vicilins are well-known storage proteins, and it has been shown that some vicilin proteins are cleaved into smaller peptides that exhibit *in vitro* antimicrobial activity (*76*, *78*). Later on an 8kDa peptide homologue to vicilin was isolated from melon fruit seeds and their antimicrobial activity has been demonstrated (*79*).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Glycine-Rich Cysteine-Free Antimicrobial Peptides

As mentioned before, most of the plant AMPs have cysteine amino acids forming disulfide bridges that stabilize a globular tertiary structure. It is then of special interest the identification of a few plant AMPs without cysteine residues. Glycine-rich AMPs are quite common in insects and it has been postulated that they may be a constitutive element of defense in plants as well (*67*).

Shepherin I and shepherin II are two AMPs of 28 and 38 amino acids, respectively, in which almost all amino acids are glycine and histidine (*80*). They were isolated from roots of shepherd's purse plants and exhibit antimicrobial activity against Gram-negative bacteria and fungi, including some pathogens of relevance in agriculture (e.g. *Erwinia herbicola* and *F. culmorum*). Contrary to almost all plant AMPs that have compact tertiary structure stabilized with disulfide bridges, shepherins have a random coil structure without any α-helices. Later, a family of novel 8 structurally different glycine-rich cysteine-free peptides were purified from wheat seeds (*67*).

Biological Function of Plant Antimicrobial Peptides

Plants have evolved to produce a large number of different AMPs as components of the innate immune system. These AMPs play an important role in the defense against microbial infection. With such large numbers, it is difficult to prove each AMP individual contribution to plant immunity by gene knockout. Nonetheless, substantial evidences confirm their function in plant defense. Among them, AMP genes are constitutively expressed in flowers and seeds, the reproductive tissues which are particularly sensitive to infection. Also, AMP gene expression is induced in vegetative tissues, both locally and systemically, in response to infection or wounding (*4*). This pathogen induced expression of AMP genes correlates with enhanced disease resistance, including systemic acquired resistance (*81*). Moreover, many of these genes have been shown to reduce the severity of disease symptoms when overexpressed in genetically engineered plants (Table I). In addition, it has been established that pathogen virulence is increased upon acquiring resistance to AMPs (*82*, *83*).

Certain plant AMPs have also a function in the defense against predatory insects. Among the known insecticidal AMPs are cyclotides and other knottin-

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

like peptides, some defensins, and some thionins (*84*). The molecular basis of the insecticidal activity of cyclotides involves the disruption of epithelial cells in the midgut of target insects through membrane binding (*85*). In contrast, other knottin-like peptides and defensins with anti-insect properties are potent inhibitors of major digestive enzymes, such as trypsin or α-amylase, that retard insect growth by impairing protein digestion (*33*).

AMPs not only combat enemies, but they also contribute to fine tune the interaction of plants with commensal and symbiotic microbial populations (*86*). For instance, in the symbiotic association of legumes with the nitrogen-fixing rhizobia, plants produce specific AMPs in the root nodules. These AMPs control the differentiation of the endosymbiont bacteria by inhibiting bacterial division and leading to cell elongation. Thus legume plants adopt effectors of the immune system to dominate their endosymbionts for their own benefit (*87*).

Certain plant AMPs might also act as regulators of innate immune response. For instance DIR1, a putative LTP, has been proposed as the translocator for release of the mobile signal in the systemic acquired resistance response in *A. thaliana* plants (*88*). In other studies, a tobacco LTP upon interaction with jasmonic acid, a defense mediating phytohormone, is shown to enhance resistance toward the pathogen *Phytophthora parasitica* (*89*).

AMPs might also play a role during abiotic stress adaptation. Evidence shows that the expression of some plant defensin, LTP, and thionins genes is induced under abiotic stress conditions (*33*, *90*). The observation that transgenic Arabidopsis plants expressing the pepper *CaLTP1* gene exhibit tolerance to salt and drought stress further supports the involvement of AMPs in the plant response to abiotic stress (*90*). Other functions have been proposed for the constitutive accumulated AMPs, as seed storage proteins in the case of thionins or defensins, or as signalling molecules in the case of defensin-like peptides during reproductive processes (*91*).

Biotechnological Applications of Plant Antimicrobial Peptides

AMPs are considered as new subsustitutes for conventional pesticides and antibiotics based on their properties: they are natural antibiotics, they show rapid and potent activity against a broad spectrum of pathogens, and they show low toxicity to the host organisms. Moreover, resistance against AMPs is rarely observed because these peptides target primary features of microbial cells. In addition, plant AMPs are highly stable to protease degradation, to heat, and to extreme pH. The application fields of AMPs include cosmetics, biomaterials, food conservation, animal feeding, biomedicine, and agriculture (*10–12*, *92*).

The application of AMPs as biopreservatives in cosmetics, biomaterials and food conservation is considered as an alternative to avoid the use of traditional chemical preservatives, given the public concern on the effects of chemical preservatives on human health and food taste (*12*). Other applications concern the development of additives for animal feeding, replacing traditional antibiotics which might damage the balance of the animal instentinal microflora and remain in livestock products upon sacrifice (*12*). Similarly, AMPs could be applied in aquaculture industry as an additive to fish feeding to enhance disease resistance and to avoid the accumulation of antibiotics. The use of transgenic algae accumulating AMPs has been proposed as an alternative strategy (*93*).

Plant AMPs have also gained attention in human health, since they display antimicrobial activity not only against plant pathogens but also against human pathogens. The discovery of the inhibitory effect toward certain types of human cancer cells of some plant defensins, LTPs, thionins, and cyclotides, opens new possibilities for cancer chemotherapy (*33*, *61*, *94*). The mechanism by which those plant AMPs inhibit proliferation of cancer cells has not been fully elucidated. The mechanism could be related to the fact that cancer cells present an aberrant high expression of anionic molecules in the outer membrane, such as sialic acid, phosphatidylserin and O-glycosylated mucins, which endow them with a more negative charge at the surface. The negative charge at the surface may act as a docking site for cationic AMPs, attracting them to the membrane where they can exert its toxic effect. The interaction between AMPs and normal cells is not favoured because of the overall neutral charge (*94*).

Certain AMPs are considered as potential therapeutical agents against the human immunodeficiency virus (HIV). For instance, some plant defensins, such as the phaseococcin, the sesquin, and the lunatusin, exhibited inhibitory activity towards HIV by curtailing the activity of the viral reverse retrotranscriptase (*94*). Plant cyclotides also display a citotoxic activity towards HIV-infected cells (*95*). The mechanism of the anti-HIV activity of cyclotides has not been determined but their selective toxicity for virus-infected cells over uninfected cells suggests that they target the membranes of virus-infected cells. It is true that cyclotides are typically active against virus-infected cells. However, the therapeutic index (i.e. the ratio of toxicity for infected cells versus normal cells) is not very high. So, new cyclotides with enhanced anti HIV-activity and reduced citotoxicity to uninfected cells need to be designed for HIV therapies (*95*).

Other studies demonstrate that some plant AMPs have antiparasitic activity against the *Leishmania donavani* promastigotes, the causative agent of human visceral Leishmaniasis (*96*). Some cyclotides are active against the human *Necator americanus,* a parasit responsible for the necatoriasis disease (*94*). These findings open new prospects for the pharmacological applications of plant AMPs as new antiparasite agents on human diseases.

The main application of plant AMPs is in agriculture. Phytopathogens are responsible for significant losses in cultivated and stored crops and are a major impediment to effective food distribution worldwide. Moreover, spoilage can increase the incidence of carcinogens (e.g. micotoxins) that affect human and animal health. To cope with this, crop protection relies mainly on chemical antimicrobials and pesticides, which significantly increase production costs and are regarded as serious environmental contaminants. Furthermore, the use of chemicals is currently under strong restrictions and regulatory requirements. AMPs are regarded as an effective alternative to chemicals for plant disease control by either transgenic expression or by topical application.

The use of AMPs to improve disease resistance by genetic engineering of crop plants will be extensively discussed in the next section. Regarding the topical application of AMPs, there are examples with experimental bioassays that are

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

close to a viable use. For instance, there is a case study of postharvest fruit disease control in which AMPs are used as a preservative (*97*). Another possibility is to use AMPs in combination with other antibiotics to obtain synergistic effects and to arrive to a more environmentally friendly practice reducing the massive use of chemicals. Topical application of AMPs is mainly limited however because there is still not a way to produce AMPs cost effectively. Yet another prospective application in agriculture is the use of genetically modified biocontrol microorganisms to produce and release AMPs (*98*, *99*).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Table I. Plant AMPs produced in transgenic plants conferring resistance to phytopathogens

AMP	*Source*	*Host*	*Pathogen*	*Ref.*
α-thionin	Barley	Tobacco	*Pseudomonas syringae*	(*107*)
Thionin2.1	*Arabidopsis*	Arabidopsis	*Fusarium oxysporum*	(*100*)
		Tomato	*Fusarium oxysporum,* *Ralstonia solanacearum*	(*108*)
Asthi1	Oat	Rice	*Burkholderia plantarii,* *Burkholderia glumae*	(*109*)
Rs-AFP2	*Raphanus sativus*	Tobacco	*Alternaria longipes*	(*36*)
		Wheat	*Fusarium graminearum,* *Rhizoctonia cerealis*	(*104*)
		Tomato	*Fusarium oxysporum,*	(*102*)
		Rice	*Botrytis cinerea* *Magnaporthe oryzae,* *Rhizoctonia solani*	(*103*)
Dm-AMP1	*Dahlia merckii*	Papaya	*Phytophthora palmivora*	(*110*)
		Rice	*Magnaporthe oryzae,* *Rhizoctonia solani*	(*111*)
		Eggplant	*Botrytis cinerea,* *Verticillium albo-atrum*	(*112*)
Wasabi defensin	*Wasabia japonica*	Rice	*Magnaporthe oryzae*	(*113*)
		Egusi melon	*Alternaria solani,* *Fusarium oxysporum*	(*114*)
Defensin BjD	Mustard	Tobacco	*Fusarium verticillioides,* *Phytophthora parasitica*	(*115*)
		Peanut	*Pheaoisariopsis personata,* *Cercospora arachidicola*	

Continued on next page.

Table I. (Continued). Plant AMPs produced in transgenic plants conferring resistance to phytopathogens

AMP	*Source*	*Host*	*Pathogen*	*Ref.*
Alf-AFP	*Medicago sativa*	Potato	*Verticillium dahliae*	(*106*)
BrD1	*Brassica rapa*	Rice	*Nilaparvata lugens*	(*105*)
NmDef02	*Nicotiana megalosiphon*	Potato	*Phytophthora parasitica*	(*116*)
Mj-AMP1	*Mirabilis jalapa*	Tomato	*Alternaria solani*	(*117*)
Mj-AMP2	*Mirabilis jalapa*	Rice	*Magnaporthe oryzae*	(*118*)
PCI	Potato	Rice	*Magnaporthe oryzae,* *Fusarium verticillioides*	(*69*)
Pn-AMP	*Pharbitis nil*	Tobacco	*Phytophthora parasítica*	(*119*)
		Tomato	*Phytophthora capsici,* *Fusarium oxysporum*	(*120*)
Ac-AMP1	*Amaranthus caudatus*	Poplar	*Septoria musiva*	(*121*)
Mi-AMP1	*Macadamia integrifolia*	Canola	*Leptosphaeria maculans*	(*122*)
Puroindolin A and B	Wheat	Rice	*Magnaporthe oryzae,* *Rhizoctonia solani*	(*123*)
		Corn	*Cochliobolus heterostrophus*	(*124*)
LTP2	Barley	Arabidopsis	*Pseudomonas syringae*	(*125*)
CaLTPI	*Capsicum annuum*	Arabidopsis	*Pseudomonas syringae,* *Botrytis cinerea*	(*90*)
Ace-AMP1	*Allium cepa*	Wheat	*Blumeria graminis*	(*126*)
		Rose	*Sphaerotheca pannosa*	(*127*)
		Geranium	*Botrytis cinerea*	(*128*)
		Rice	*Magnaporther oryzae,* *Rhizoctonia solani,* *Xanthomonas oryzae*	(*129*)
Snakin1	*Solanum chacoense*	Potato	*Rhizoctonia solani* *Erwinia carotovora*	(*130*)
Snakin2	Tomato	Tomato	*Clavibacter michiganensis*	(*131*)

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Plant Disease Protection by Transgenic Expression of AMP Genes

Multiple reports can be found in the literature on the expression of genes encoding AMPs in model and crop plant species conferring different degrees of protection against fungal and/or bacterial pathogens. A summary is presented in Tables I-III. Several approaches based on different AMP sources will be reviewed in the next paragraphs.

Plant Protection by Transgenic Expression of Plant AMP Genes

A reduced number of reports show phenotypes of pathogen resistance in transgenic plants that overexpress endogenous AMP genes (*100*, *101*). These works were approached in the model plant *A. thaliana* by overexpression thionin (*Thi2.1*) and defensin (*pdf1.1*) genes, demonstrating a direct role of these genes in plant defense. However, the level of disease protection conferred by overexpression of the AMP genes in the plant of origin was limited and not very efficient against aggressive pathogens.

Conversely, the heterologous expression of genes encoding plant AMPs has been reported to confer enhanced resistance towards bacterial and fungal pathogens in model, crop and ornamental transgenic plants (Table I). Successful strategies were based on genes that encode thionin, defensin, LTP, hevein-like, snakins, and β-barrelin peptides (Table I). For instance, the *Raphanus sativa Rs-AFP2* gene encoding a defensin was transferred into the tobacco model plant (*36*), and the crop plants tomato (*102*), rice (*103*), and wheat (*104*), and proven as an efficient strategy to increase resistance to fungal pathogens. Noteworthy, the insecticidal activity of the *Brassica rapa* defensin conferred resistance against the brown planthopper in transgenic rice plants (*105*). One of the first examples of disease protection under field conditions was reported in potato plants by transgenic expression of the alfalfa antifungal (alfAFP) gene increasing resistance against *V. dahliae* to levels that are equal to or that exceed those obtained through current practices based on fumigants (*106*).

Plant Disease Protection by Transgenic Expression of Non-Plant AMP Genes

Most of the strategies based on the use of natural plant antimicrobial genes for plant genetic engineering have been fairly narrow with respect to the microbial spectrum of protection. Due to coevolutionary aspects, antimicrobial peptides from non-plant origin could potentially be more effective against plant pathogens. Some examples are presented in Table II, including the use of the insect antimicrobial peptides cecropins, attacins, and apidecins, or the frog peptides magainins and temporins. The success of such strategies depends on the efficient expression of the transgene in the host plant, the stability of the AMPs in plant tissues, and their potency as antimicrobial agents. This aspect can be illustrated, as explained next, with several attempts to enhance resistance through the expression of genes encoding cecropins or analogs, which produced contradictory results regarding pathogen resistance.

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

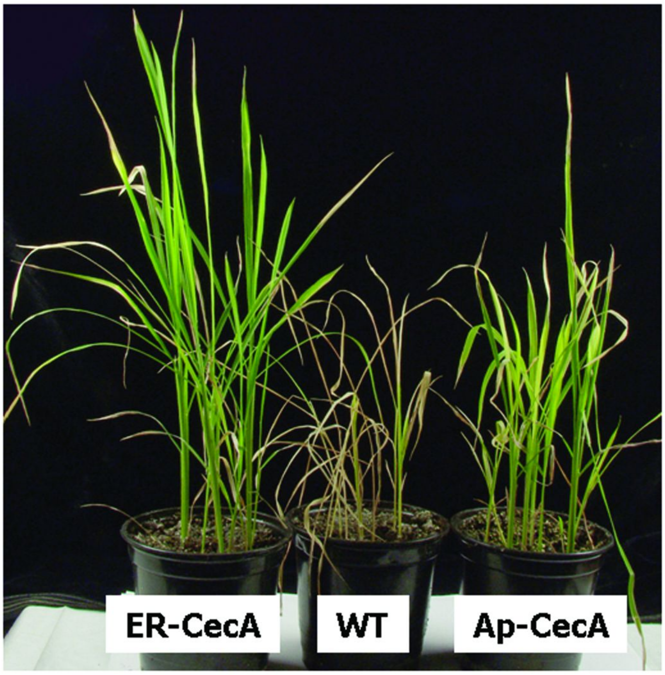


Figure 2. Blast resistance of transgenic rice plants expressing the cecropin A gene.Phenotype of wild-type (WT) and transgenic plants accumulating the cecropin A peptide in the apoplast (Ap-CecA) or in the endoplasmic reticulum (ER-CecA) 30 days after inoculation with M. oryzae spore suspension (10^4 spores/ml).

Cecropins are α-helical peptides isolated from the hemolymph of insects that possess lytic activity against bacterial and fungal phytopathogens, but they did not show lytic activity against plant and animal cells (*132*). These characteristics convert cecropin peptides in potential tools for developing disease resistance in plants. The production of cecropin peptides in transgenic plants requires the synthesis of genes with a codon usage adapted to the host plants to guarantee a good level of the transgene expression. Synthetic cecropin genes have been introduced in several plant species producing differential results. Although, disease symptoms were reduced upon *Xanthomonas oryzae* infection in rice plants producing the cecropin B peptide (*133*), no enhanced resistance to bacterial pathogens was observed in tobacco plants producing the same peptide (*134*). The failure to confer protection of cecropin peptides was attributed to their differential susceptibility to degradation by host proteases, which varies from one plant species to another (*135*, *136*). Thus, the success of these strategies might depend on the accumulation of cecropin peptides in different subcellular compartments to be protected from host proteases. As an example, the production of cecropin A in rice plants confers protection to fungal pathogen when peptide is accumulated either in the extracellular space or in the endoplasmic reticulum (*137*). The cecropin A rice plants showed a broad spectrum protection against different fungal pathogens, such as *M. oryzae* (Figure 2) or *Fusarium verticillioides* (M. Coca, unpublished results). These results show the potential of the cecropin AMPs to engineer disease resistance in plants when protected from intracellular protease degradation.

Another remarkable example based on AMPs of non-plant origin is the introduction of the insect *attacin E* gene in the "Royal Gala" apple tree that resulted in significant resistance to the bacterial pathogen *Erwinia amylovora*, the causal agent of the fire blight disease (*138*). This strategy has proven efficient conferring stable resistance throughout 12 year periods of orchard growth of the transgenic apple trees (*139*).

The genome of mycoparasitic and antagonistic fungi, which has evolved specifically to attack other fungi but not plants, represents a potential source of AMP genes to engineering fungal resistance in plants. An example is the antifungal peptide (AFP) isolated from the mold *Aspergillus giganteus*. The AFP structure resembles that of the plant defensins and γ-thionins and shows a potent inhibitory activity against phytopathogenic fungi (*140*). Transgenic rice plants constitutively expressing the gene encoding the AFP showed enhanced resistance to the rice blast fungus *M. oryzae* (*141*).

Table II. Non-plant AMPs produced in transgenic plants conferring resistance to phytopathogens

AMP	*Source*	*Host*	*Pathogen*	*Ref.*
hBD2	Human	Arabidopsis	*Botrytis cinerea*	(*142*)
CecropinP1	Ascaris nematodes	Tobacco	*Pseudomonas syringae Pseudomonas marginata* Erwinia carotovora	(*143*)
Cecropin A	*Hyalophora cecropia*	Rice	*Magnaporthe oryzae*	(*137*)
Cecropin B	*Bombyx mori*	Rice	*Xanthomonas oryzae*	(*133*)
Cecropin B	*Hyalophora cecropia*	Tomato	*Ralstonia solanacearum* *Xanthomonas campestris*	(*144*)
Attacin E	*Hyalophora cecropia*	Apple	*Erwinia amylovora*	(*138*)
Attacin A	*Trichoplusia ni*	Orange	*Xanthomonas citri*	(*145*)
Metchnikowin	*Drosophila melanogaster*	Barley	*Fusarium graminearum* *Blumeria graminis*	(*146*)

Continued on next page.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Table II. (Continued). Non-plant AMPs produced in transgenic plants conferring resistance to phytopathogens

AMP	*Source*	*Host*	*Pathogen*	*Ref.*
Drosopmycin	*Drosophila melanogaster*	Tobacco	*Cercospora nicotaniae*	(*147*)
Gallerimycin	*Galleria mellonella*	Tobacco	*Erysiphe cichoracearum* *Sclerotinia minor*	(*148*)
Sarcotoxin IA	*Sarcophaga peregrina*	Tobacco	*Pseudomonas syringae* *Erwinia carotovora*	(*149*)
Heliomicin	*Heliothis virescens*	Tobacco	*Cercospora nicotaniae*	(*147*)
Thanatin	*Podisus maculiventris*	Rice	*Magnaporthe oryzae*	(*150*)
Tachyplesin	Horseshoe crab	Potato	*Erwinia carotovora*	(*151*)
Esculentin1	Rana esculenta	Tobacco	*Pseudomonas syringae* *Pseudomonas aeruginosa* *Phytophthora nicotianae*	(*152*)
Temporin A	Rana temporaria	Tobacco	Bacteria, fungi, oomycetes	(*153*)
Mussel defensin	Mussel	Tobacco	*Pseudomonas syringae*	(*154*)
AFP	*Aspergillus giganteous*	Rice Wheat	*Magnaporthe oryzae* *Erysiphe graminis* *Puccinia recondita*	(*141*, *155*) (*156*)

Plant Disease Protection by Transgenic Expression of Synthetic AMP Genes

Molecular modeling and engineering of peptides provides a powerful tool to generate chimeric peptides with potentially superior properties, including less susceptibility to plant proteases and less hemolytic activity. Genes encoding several synthetic AMPs have been also transferred to plants to confer resistance against phytopathogens (Table III). A sucessfull example of this approach is the synthetic peptide MsrA1, a cecropin-melittin chimera, with broad-spectrum antimicrobial activity. Transgenic potato plants expressing the *MsrA1* gene exhibit broad-spectrum resistance against bacterial and fungal pathogens, and tubers retained their resistance to infectious challenge for more than a year. Absence of toxicity was inferred by feeding mice with the transgenic potato tubers (*160*). Another example is found with the synthetic gene *MsrA3*, encoding a modified analog of temporin A, which confers protection to potato diseases, while simultaneously prevents storage losses of tubers (*162*). Other significant examples of synthetic peptides are D4E1 and MSI-99. Their effectiveness in various plant species by conferring protection against different pathogens has been demonstrated (Table III). In addition to antibacterial and antifungal protection, there are reports for antiviral protection conferred by indolicidin and polyphemusin variants when produced in tobacco plants (*172*, *173*).

Table III. Synthetic AMPs produced in transgenic plants conferring resistance to phytopathogens

AMP	*Source*	*Host*	*Pathogen*	*Ref.*
Shiva-1	Cecropin B-analog	Tobacco	*Pseudomonas solanacearum*	(*157*)
Pep11	Cecropin A-derivative	Tomato	*Phytophthora infenstans*	(*158*)
CEMA	Cecropin-Melittin chimera	Tobacco	*Fusarium solani*	(*159*)
MsrA1	Cecropin-Melittin chimera	Potato	*Erwinia carotovora* *Phytophthora cactorum* *Fusarium solani*	(*160*)
MsrA2	Dermaseptin B1 derivative	Potato Tobacco	*Erwinia carotovora*, fungi Bacteria, fungi, oomycetes	(*161*) (*153*)
MsA3	Temporin A derivative	Potato	*Erwinia carotovora*, *Phytophthora infenstans*, *Phytophthora erythroseptica*	(*162*)
D4E1	Synthetic	Tobacco Poplar Cotton	*Colletotrichum destructivum* *Agrobacterium tumefaciens*, *Xanthomonas populi* *Thielaviopsis basicola*	(*163*) (*164*) (*165*)
MSI-99	Magainin-analog	Tobacco Tobacco-chloroplasts	Bacteria, fungi *Pseudomonas syringae*, *Colletotrichum destructivum*	(*166*) (*167*)
		Banana	*Fusarium oxysporum*, *Mycosphaerella musicola*	(*166*)
		Grapevine	*Agrobacterium vitis*, *Uncinula necator*	(*168*)
		Tomato	*Pseudomonas syringae*	(*169*)
Myp30	Magainin-analog	Tobacco	*Pseudomonas tabacina*, *Erwinia carotovora*	(*170*)
ESF12	Magainin-analog	Poplar	*Septoria musiva*	(*121*)
Rev4	Indolicin-variant	Tobacco Arabidopsis	Bacteria, oomycetes	(*171*)

Continued on next page.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Table III. (Continued). Synthetic AMPs produced in transgenic plants conferring resistance to phytopathogens

AMP	*Source*	*Host*	*Pathogen*	*Ref.*
10R 11R	Indolicin variants	Tobacco	TMV, *Erwinia carotovora*, *Botrytis cinerea*, *Verticillium* sp.	(*172*)
PV5	Polyphemusin variant	Tobacco	TMV, *Erwinia carotovora*, fungi	(*173*)
ACHE-I-7.1	Synthetic	Potato	*Globodera pallida*	(*174*)
ESF39A	Synthetic	Elm	*Ophiostoma novo- ulmi*	(*175*)

Plant Disease Protection by Inducible Expression of AMP Genes

The simplest mean to genetic engineering resistance to phytopathogens entails the constitutive expression of the genes encoding the AMPs in plants. Even though this strategy is suitable as proof-of-concept to assess the effectiveness of the transgene expression, it presents a number of potential drawbacks for actual use in genetically improved crops. Among them is a potential negative impact on fitness and yield in the host plant, or the selection of resistant populations of target pathogens. Instead, a controlled production of the AMP in the transgenic plant represents a more desirable strategy for protection of crop species against pathogens. In this way, the AMP will be produced at the site where it is needed and only when needed. This strategy can be accomplished by the use of pathogen-inducible promoters to drive the expression of AMP genes. In addition, it will be desirable the use of promoters not active in edible organs to avoid the accumulation of AMPs in the organs used for human and animal consumption. There are several reports on the controlled production of AMPs in genetically modified plants. For instance, the pathogen-inducible expression of the *AFP* gene from *A. giganteous* in rice plants was reported to confer protection against rice blast disease (*155*). The expression of the AFP-encoding gene was driven by the maize *ZmPR4* gene promoter, which was quickly and strongly activated in rice leaves in response to pathogen infection. Moreover, the *ZmPR4* promoter was not active in the rice endosperm, the edible organ of the plant. The level of protection conferred by the inducible expression of the *AFP* gene in rice was superior to that observed in transgenic rice constitutively expressing the same gene (*141*).

Another example is the expression of the antifungal insect peptide metchnikowin, under the control of the bacterial pathogen- and wound- inducible mannopine synthase promoter in transgenic wheat plants to improve resistance to fungal pathogens (*146*). Similarly, a wound-inducible promoter was used to drive the expression in apple trees of the insect gene encoding the peptide attacin to improve resistance to fire blight (*139*). In this case, the used promoter of the potato proteinase inhibitor II gene was used. This promoter showed a low level of expression in apple, a very convenient strategy to avoid taste changes on fruits.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Additional Aspests on the Transgenic Expression of AMP Genes

Several aspects require to be carefully considered when producing AMPs in transgenic plants. One is the impact of AMP production on beneficial microorganisms to the host plant, like mycorrhizal fungi. This aspect has been often neglected, and only few studies regarding the effect of the transgene expression on mycorrhizae have been reported. As an example, eggplants (*Solanum melongena*) constitutively expressing a natural AMP, the dahlia defensin DmAMP1, showed resistance to pathogenic *B. cinerea* and *Verticillium* sp, while symbiosis with the arbuscular mycorrhizal fungus *Glomus mosseae* was not significantly affected (*112*). In a field study with transgenic elm trees producing a synthetic antimicrobial peptide, it was shown that mycorrhizal colonization was similar to that of the wild-type trees (*175*). In this case, the expression of the antimicrobial gene was driven by a vascular promoter and mycorrhiza could be not exposed to the AMP. This example again points to the convenience of regulating the expression of *AMP* genes in engineered plants.

Although there are multiple studies on the benefits of transgene approaches to enhance protection against pathogens, studies on the transgene induced changes in the host plants are still scarce. In this respect, transcriptomic analysis of cecropin A-expressing rice plants showed that the accumulation of the peptide has an impact on host gene expression (*176*). Among the up-regulated genes in cecropin A plants are genes involved in protection against oxidative stress, which are known to be required for pathogen resistance. These results suggest that fungal resistance might be the consequence of a combination of the antifungal activity of cecropin A and *cecropin A*-mediated overexpression of rice genes. Approaching this type of studies is very relevant to understand the substantial equivalence between transgenic and wild-type plants.

Biotechnological Approaches for Production of Antimicrobial Peptides

One of the main barriers that might impede the development of AMPs as commercial therapeutic agents, or restrict their applicability as additives in plant protection, is their high production costs. Although possible, the chemical synthesis of these peptides is very expensive. Biotechnological procedures using microbial systems or transgenic plants as biofactories for production of AMPs might help to solve these challenges. The short sequence length of AMPs makes feasible the design of synthetic genes for their heterologous production. Several attempts have been made to produce AMPs using bacteria, fungi, or plant based systems which have proven to be commercially feasible to date.

Prokaryotic expression of the cysteine-rich plant AMPs is always a challenge, mainly due to the improper disulfide bridge formation in the high reducing cytoplasm of the common expression host strains, the easy degradation, and the toxicity to the host. Only few successful examples can be found in the literature, using approaches based on the production of fusion proteins to glutathione-S-transferase (*177*) or to thioredoxin (*178*). Another microbial system widely used for recombinant protein production is *Pichia pastoris* (*179*). Recently, the cystein-rich antifungal peptide AFP from *A. giganteus* has been successfully produced in *P. pastoris* with yields of milligrams per liter of culture (*180*). The AFP recombinant protein shows structural and antifungal properties comparable to the *Aspergillus* produced AFP.

The most promising AMP-production platforms are plant-based systems, since there is consistent evidence that AMP-producing transgenic plants can be obtained (Tables I-III). Plants provide a safe, easily scalable, and cheap system for large production of AMPs. Several plant-based production platforms can be considered, ranging from seed- and leaf-based production in stable transgenic plant lines to plant cell bioreactors, or to viral or Agrobacterium-mediated transient expression systems. Each system has advantages and drawbacks. The choice depends on the crop, the peptide and its application.

Seed-based production is a convenient system because plant AMPs are naturally accumulated in seeds, so it is possible to accumulate them in seeds without affecting the growth and development of the plant. Although seed-based production is slower than transient expression systems in providing the initial material, seeds possess the optimal biochemical environment for a long-term stable storage of AMPs, with the advantage that production can be decoupled from the extraction and purification processes. An important factor to consider for the production of AMPs in plant tissues is subcellular compartmentation which may have a major effect in the level of AMP accumulation. Additionally, compartmentation of AMPs into specific subcellular organelles can protect AMPs from protease degradation and facilitate their purification process. The process that highly increases the production costs of the recombinant peptides in plant-based systems is the purification of the products, but different degrees of purification are required depending on the intended use for these peptides. For instance, highly purified peptides are required for medical use, but applications on crop or postharvest protection require simpler purification schemes, as simple as pulverization of seed material. In spite all these advantages seed-based systems for the production of plant AMPs have not been reported, even when, for the production of therapeutic proteins at high levels, including insulin, human growth hormone, lysozyme and the antimicrobial protein lactoferrin they have been successfully used (*181*).

Another promising system is the use of chloroplasts as bioreactors for large-scale economic production of AMPs. Chloroplast-based production offers several advantages, including high levels of transgene expression, transgene containment via maternal inheritance, and multi-gene expression in a single transformation event. Several antibiotics have been produced in chloroplasts, including the magainin analog MSI-99 (*167*), the PhyGBS lysine (*182*), and the retrocyclin-101 and protegrin-1 AMPs (*183*). Finally, for the high value cyclotides peptides, plant cell culture systems represent the best option for producing active cyclotides in qualities and quantities required for therapeutic applications (*184*).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Conclusions and Future Challenges

Antimicrobial peptides are evolutionary conserved components of the innate immune system found among all classes of life, bacteria, plants, animals and man. Theses peptides are excellent candidates for development as novel therapeutic agents and complements to conventional antibiotic therapy. Thus in contrast to conventional antibiotics they generally have a broad range of activity and require a short contact time to induce killing with little opportunity for development of resistance. In view of the increasing resistance by microorganisms to conventional antibiotics, these unique natural agents have the potential of being applied in multiple situations, such as crop protection, food preservation or human health.

This review highlights the implication of AMPs in the plant defense response to pathogen infection. However, the examples presented here probably represent only the tip of the iceberg. Discovery of novel plant AMPs would give us an evolutionary insight into why certain gene families expanded in plants while others are absent. Whereas there is compelling evidence that AMPs play a key role in plant protection against pathogen infection, the application of antimicrobial peptides in agriculture is still in its infancy. One of the most obvious challenges for the future is to develop efficient and cost-effective alternatives for the production of AMPs and their subsequent application for crop protection. For exploitation in agriculture, the future challenge is to find distint potent antimicrobial peptides that target relevant pathogens. Still several issues need to be addressed for the biotechnological production of AMPs in plants, including intrinsic toxicity to plant and animal cells. Transgenically produced antimicrobial peptides should be directed to the relevant plant tissues, cell types and subcelular compartments, and peptide stability and proper folding need to be considered. It is anticipated that combinations of potent antimicrobial peptides will provide agronomically relevant levels of disease control and should contribute to more sustainable agricultural practices.

Acknowledgments

This work was supported by grant EUI2008-03769, BIO2009-08719 and AGL2010-16847 from the Spanish Ministry of Science and Innovation as well as by the Consolider-Ingenio 2010 Programme CSD2007-00036. We also thank the “Department d´Innovació, Universitats i Empresa” from the Generalitat de Catalunya (Xarxa de Referencia en Biotecnología and SGR 09626) for substantial support.

References

1. Jones, J. D. G.; Dangl, J. L. *Nature* **2006**, *444*, 323–329.
2. Boller, T.; He, S. Y. *Science* **2009**, *324*, 742–744.
3. Flor H. H. *Annu. Rev. Phytopathol.* **1971**, 9, 275.
4. Sels, J.; Mathys, J.; De Coninck, B. M. A.; Cammue, B. P. A.; De Bolle, M. F. C. *Plant Physiol. Biochem.* **2008**, *46*, 941–950.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

5. Van Loon, L. C.; Rep, M.; Pieterse, C. M. J. *Annu. Rev. Phytopathol.* **2006**, *44*, 135–162.
6. Benko-Iseppon, A. M.; Galdino, S. L.; Calsa, T.; Kido, E. A.; Tossi, A.; Belarmino, L. C.; Crovella, S. *Curr. Protein Pept. Sci.* **2010**, *11*, 181–188.
7. Silverstein, K. A. T.; Moskal, W. A.; Wu, H. C.; Underwood, B. A.; Graham, M. A.; Town, C. D.; VandenBosch, K. A. *Plant J.* **2007**, *51*, 262–280.
8. Zasloff, M. *Nature* **2002**, *415*, 389–395.
9. Hancock, R. E. W.; Sahl, H. G. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.
10. Montesinos, E. *FEMS Microbiol. Lett.* **2007**, *270*, 1–11.
11. Marcos, J. F.; Muñoz, A.; Pérez-Payá, E.; Misra, S.; López-García, B. *Annu. Rev. Phytopathol.* **2008**, *46*, 273–301.
12. Keymanesh, K.; Soltani, S.; Sardari, S. *World J. Microbiol. Biotechnol.* **2009**, *25*, 933–944.
13. Brogden, K. A. *Nat. Rev. Microbiol.* **2005**, *3*, 238–250.
14. Thomas, S.; Karnik, S.; Barai, R. S.; Jayaraman, V. K.; Idicula-Thomas, S. *Nucl. Acids Res.* **2010**, *38*, D774–D780.
15. Hammami, R.; Ben Hamida, J.; Vergoten, G.; Fliss, I. *Nucl. Acids Res.* **2009**, *37*, D963–D968.
16. Wang, G. S.; Li, X.; Wang, Z. *Nucl. Acids Res.* **2009**, *37*, D933–D937.
17. Harris, F.; Dennison, S. R.; Phoenix, D. A. *Curr. Protein Pept. Sci.* **2009**, *10*, 585–606.
18. Padovan, L.; Scocchi, M.; Tossi, A. *Curr. Protein Pept. Sci.* **2010**, *11*, 210–219.
19. Pelegrini, P. B.; del Sarto, R. P.; Silva, O. N.; Franco, O. L.; Grossi-de-Sa, M. F. *Biochem. Res. Int.* **2011**, *2011*, 1–9.
20. Marcos, J. F.; Gandia, M. *Expert Opin. Drug. Discovery* **2009**, *4*, 659–671.
21. Thevissen, K.; Warnecke, D. C.; François, I. E. J. A.; Leipelt, M.; Heinz, E.; Ott, C.; Zahringer, U.; Thomma, B. P. H. J.; Ferkel, K. K. A.; Cammue, B. P. A. *J. Biol. Chem.* **2004**, *279*, 3900–3905.
22. Regente, M. C.; Giudici, A. M.; Villalaín, J.; de la Canal, L. *Lett. Appl. Microbiol.* **2005**, *40*, 183–189.
23. Thevissen, K.; Terras, F. R. G.; Broekaert, W. F. *Appl. Environ. Microbiol.* **1999**, *65*, 5451–5458.
24. Henriques, S. T.; Melo, M. N.; Castanho, M. A. R. B. *Biochem. J.* **2006**, *399*, 1–7.
25. Muñoz, A.; López-García, B.; Marcos, J. F. *Antimicrob. Agents Chemother.* **2006**, *50*, 3847–3855.
26. Moreno, A. B.; Martínez del Pozo, A.; San Segundo, B. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 883–895.
27. Schraderfischer, G.; Apel, K. *Mol. Gen. Genet.* **1994**, *245*, 380–389.
28. Bohlmann, H.; Apel, K. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 227–240.
29. Molina A.; Ahl-Goy P.; Fraile A.; Sánchez-Monge R.; García-Olmedo F. *Plant Sci.* **1993**, *92*, 177.
30. Pineiro, M.; Diaz, I.; Rodríguez-Palenzuela, P.; Titarenko, E.; García-Olmedo, F. *FEBS Lett.* **1995**, *369*, 239–242.

31. Carrasco, L.; Vazquez, D.; Hernandezlucas, C.; Carbonero, P.; Garciaolmedo, F. *Eur. J. Biochem.* **1981**, *116*, 185–189.
32. Cammue, B. P. A.; Debolle, M. F. C.; Terras, F. R. G.; Proost, P.; Vandamme, J.; Rees, S. B.; Vanderleyden, J.; Broekaert, W. F. *J. Biol. Chem.* **1992**, *267*, 2228–2233.
33. Carvalho, A. D.; Gomes, V. M. *Peptides* **2009**, *30*, 1007–1020.
34. Thomma, B. P. H. J.; Cammue, B. P. A.; Thevissen, K. *Planta* **2002**, *216*, 193–202.
35. Bruix, M.; Jimenez, M. A.; Santoro, J.; Gonzalez, C.; Colilla, F. J.; Mendez, E.; Rico, M. *Biochemistry* **1993**, *32*, 715–724.
36. Terras, F. R. G.; Eggermont, K.; Kovaleva, V.; Raikhel, N. V.; Osborn, R. W.; Kester, A.; Rees, S. B.; Torrekens, S.; Van Leuven, F.; Vanderleyden, J.; Cammue, B. P. A.; Broekaert, W. F. *Plant Cell* **1995**, *7*, 573–588.
37. Do, H. M.; Chul Lee, S.; Won Jung, H.; Hoon Sohn, K.; Kook Hwang, B. *Plant Sci.* **2004**, *166*, 1297–1305.
38. Terras, F. R. G.; Torrekens, S.; Van Leuven, F.; Osborn, R. W.; Vanderleyden, J.; Cammue, B. P. A.; Broekaert, W. F. *FEBS Lett.* **1993**, *316*, 233–240.
39. Yeats, T. H.; Rose, J. K. C. *Protein Sci.* **2008**, *17*, 191–198.
40. Samuel, D.; Liu, Y. J.; Cheng, C. S.; Lyu, P. C. *J. Biol. Chem.* **2002**, *277*, 35267–35273.
41. Lascombe, M. B.; Bakan, B.; Buhot, N.; Marion, D.; Blein, J. P.; Larue, V.; Lamb, C.; Prange, T. *Protein Sci.* **2008**, *17*, 1522–1530.
42. Carvalho, A. D.; Teodoro, C. E. D.; Da Cunha, M.; Okorokova-Facanha, A. L.; Okorokov, L. A.; Fernandes, K. V. S.; Gomes, V. M. *Physiol. Plant.* **2004**, *122*, 328–336.
43. Thoma, S.; Kaneko, Y.; Somerville, C. *Plant J.* **1993**, *3*, 427–436.
44. Dubreil, L.; Gaborit, T.; Bouchet, B.; Gallant, D. J.; Broekaert, W. F.; Quillien, L.; Marion, D. *Plant Sci.* **1998**, *138*, 121–135.
45. Regente, M. C.; de la Canal, L. *Physiol. Plant.* **2000**, *110*, 158–163.
46. Cammue, B. P. A.; Thevissen, K.; Hendriks, M.; Eggermont, K.; Goderis, I. J.; Proost, P.; Vandamme, J.; Osborn, R. W.; Guerbette, F.; Kader, J. C.; Broekaert, W. F. *Plant Physiol.* **1995**, *109*, 445–455.
47. Segura, A.; Moreno, M.; Madueño, F.; Molina, A.; García-Olmedo, F. *Mol. Plant-Microbe Interact.* **1999**, *12*, 16–23.
48. Mao, Z. M.; Zheng, J. Y.; Wang, Y. S.; Chen, G. H.; Yang, Y. H.; Feng, D. X.; Xie, B. Y. *Phytoparasitica* **2011**, *39*, 151–164.
49. Berrocal-Lobo, M.; Segura, A.; Moreno, M.; López, G.; García-Olmedo, F.; Molina, A. *Plant Physiol.* **2002**, *128*, 951–961.
50. Vanparijs, J.; Broekaert, W. F.; Goldstein, I. J.; Peumans, W. J. *Planta* **1991**, *183*, 258–264.
51. Koo, J. C.; Lee, S. Y.; Chun, H. J.; Cheong, Y. H.; Choi, J. S.; Kawabata, S.; Miyagi, M.; Tsunasawa, S.; Ha, K. S.; Bae, D. W.; Han, C. D.; Lee, B. L.; Cho, M. J. *Biochim. Biophys. Acta* **1998**, *1382*, 80–90.
52. Fujimura, M.; Minami, Y.; Watanabe, K.; Tadera, K. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1636–1642.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

53. Broekaert, W. F.; Marien, W.; Terras, F. R. G.; De Bolle, M. F.; Proost, P.; Van Damme, J.; Dillen, L.; Claeys, M.; Rees, S. B.; Vanderleyden, J. *Biochemistry* **1992**, *31*, 4308–4314.
54. Nielsen, K. K.; Nielsen, J. E.; Madrid, S. M.; Mikkelsen, J. D. *Plant Physiol.* **1997**, *113*, 83–91.
55. Lipkin, A.; Anisimova, V.; Nikonorova, A.; Babakov, A.; Krause, E.; Bienert, M.; Grishin, E.; Egorov, T. *Phytochemistry* **2005**, *66*, 2426–2431.
56. Huang, R. H.; Xiang, Y.; Liu, X. Z.; Zhang, Y.; Hu, Z.; Wang, D. C. *FEBS Lett.* **2002**, *521*, 87–90.
57. Van den Bergh, K. P. B.; Proost, P.; Van Damme, J.; Coosemans, J.; van Damme, E. J. M.; Peumans, W. J. *FEBS Lett.* **2002**, *530*, 181–185.
58. Huang, R. H.; Xiang, Y.; Tu, G. Z.; Zhang, Y.; Wang, D. C. *Biochemistry* **2004**, *43*, 6005–6012.
59. Craik, D. J.; Clark, R. J.; Daly, N. L. *Expert Opin. Invest. Drugs* **2007**, *16*, 595–604.
60. Gracy, J.; Le-Nguyen, D.; Gelly, J. C.; Kaas, Q.; Heitz, A.; Chiche, L. *Nucl. Acids Res.* **2008**, *36*, D314–D319.
61. Ireland, D. C.; Clark, R. J.; Daly, N. L.; Craik, D. J. *J. Nat. Prod.* **2010**, *73*, 1610–1622.
62. Wang, C. K. L.; Kaas, Q.; Chiche, L.; Craik, D. J. *Nucl. Acids Res.* **2008**, *36*, D206–D210.
63. Colgrave, M. L.; Craik, D. J. *Biochemistry* **2004**, *43*, 5965–5975.
64. Ireland, D. C.; Colgravel, M. L.; Nguyencong, P.; Daly, N. L.; Craik, D. J. *J. Mol. Biol.* **2006**, *357*, 1522–1535.
65. Trabi, M.; Craik, D. J. *Plant Cell* **2004**, *16*, 2204–2216.
66. Gao, G. H.; Liu, W.; Dai, J. X.; Wang, J. F.; Hu, Z.; Zhang, Y.; Wang, D. C. *Biochemistry* **2001**, *40*, 10973–10978.
67. Egorov, T. A.; Odintsova, T. I.; Pukhalsky, V. A.; Grishin, E. V. *Peptides* **2005**, *26*, 2064–2073.
68. Janssen, B. J. C.; Schirra, H. J.; Lay, F. T.; Anderson, M. A.; Craik, D. J. *Biochemistry* **2003**, *42*, 8214–8222.
69. Quilis, J.; Meynard, D.; Vila, L.; Aviles, F. X.; Guiderdoni, E.; San Segundo, B. *Plant Biotechnol. J.* **2007**, *5*, 537–553.
70. Marcus, J. P.; Goulter, K. C.; Green, J. L.; Harrison, S. J.; Manners, J. M. *Eur. J. Biochem.* **1997**, *244*, 743–749.
71. McManus, A. M.; Nielsen, K. J.; Marcus, J. P.; Harrison, S. J.; Green, J. L.; Manners, J. M.; Craik, D. J. *J. Mol. Biol.* **1999**, *293*, 629–638.
72. Pantoja-Uceda, D.; Shewry, P. R.; Bruix, M.; Tatham, A. S.; Santoro, J.; Rico, M. *Biochemistry* **2004**, *43*, 6976–6986.
73. de Souza Candido, E.; Pinto, M. F. S.; Pelegrini, P. B.; Lima, T. B.; Silva, O. N.; Pogue, R.; Grossi-de-Sa, M. F.; Franco, O. L. *FASEB J.* **2011**.
74. Duvick, J. P.; Rood, T.; Rao, A. G.; Marshak, D. R. *J. Biol. Chem.* **1992**, *267*, 18814–18820.
75. Tailor, R. H.; Acland, D. P.; Attenborough, S.; Cammue, B. P. A.; Evans, I. J.; Osborn, R. W.; Ray, J. A.; Rees, S. B.; Broekaert, W. F. *J. Biol. Chem.* **1997**, *272*, 24480–24487.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

76. Marcus, J. P.; Green, J. L.; Goulter, K. C.; Manners, J. M. *Plant J.* **1999**, *19*, 699–710.
77. De Lucca, A. J.; Jacks, T. J.; Broekaert, W. J. *Mycopathologia* **1998**, *144*, 87–91.
78. Wang, X.; Thomas, R. S.; Carroll, J. A.; Duffin, K. L. *Biochem. Biophys. Res. Commun.* **2002**, *292*, 236–242.
79. Ribeiro, S. F. F.; Agizzio, A. P.; Machado, O. L. T.; Neves-Ferreira, A. G. C.; Oliveira, M. A.; Fernandes, K. V. S.; Carvalho, A. O.; Perales, J.; Gomes, V. M. *Sci. Hortic. (Amsterdam, Neth.)* **2007**, *111*, 399–405.
80. Park, C. J.; Park, C. B.; Hong, S. S.; Lee, H. S.; Lee, S. Y.; Kim, S. C. *Plant Mol. Biol.* **2000**, *44*, 187–197.
81. Maleck, K.; Levine, A.; Eulgem, T.; Morgan, A.; Schmid, J.; Lawton, K. A.; Dangl, J. L.; Dietrich, R. A. *Nat. Genet.* **2000**, *26*, 403–410.
82. Llama-Palacios, A.; López-Solanilla, E.; Poza-Carrión, C.; García-Olmedo, F.; Rodríguez-Palenzuela, P. *Mol. Microbiol.* **2003**, *49*, 347–357.
83. López-Solanilla, E.; García-Olmedo, F.; Rodríguez-Palenzuela, P. *Plant Cell* **1998**, *10*, 917–924.
84. Gruber, C. W.; Cemazar, M.; Anderson, M. A.; Craik, D. J. *Toxicon* **2007**, *49*, 561–575.
85. Barbeta, B. L.; Marshall, A. T.; Gillon, A. D.; Craiks, D. J.; Anderson, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 1221–1225.
86. Maroti, G.; Kereszt, A.; Kondorosi, E.; Mergaert, P. *Res. Microbiol.* **2011**, *162*, 363–374.
87. Van de Velde, W.; Zehirov, G.; Szatmari, A.; Debreczeny, M.; Ishihara, H.; Kevei, Z.; Farkas, A.; Mikulass, K.; Nagy, A.; Tiricz, H.; Satiat-Jeunemaitre, B.; Alunni, B.; Bourge, M.; Kucho, K.; Abe, M.; Kereszt, A.; Maroti, G.; Uchiumi, T.; Kondorosi, E.; Mergaert, P. *Science* **2010**, *327*, 1122–1126.
88. Maldonado, A. M.; Doerner, P.; Dixon, R. A.; Lamb, C. J.; Cameron, R. K. *Nature* **2002**, *419*, 399–403.
89. Buhot, N.; Gomes, E.; Milat, M. L.; Ponchet, M.; Marion, D.; Lequeu, J.; Delrot, S.; Coutos-Thevenot, P.; Blein, J. P. *Mol. Biol. Cell* **2004**, *15*, 5047–5052.
90. Jung, H. W.; Kim, K. D.; Hwang, B. K. *Planta* **2005**, *221*, 361–373.
91. Okuda, S.; Tsutsui, H.; Shiina, K.; Sprunck, S.; Takeuchi, H.; Yui, R.; Kasahara, R. D.; Hamamura, Y.; Mizukami, A.; Susaki, D.; Kawano, N.; Sakakibara, T.; Namiki, S.; Itoh, K.; Otsuka, K.; Matsuzaki, M.; Nozaki, H.; Kuroiwa, T.; Nakano, A.; Kanaoka, M. M.; Dresselhaus, T.; Sasaki, N.; Higashiyama, T. *Nature* **2009**, *458*, 357–U122.
92. Zhang, L. J.; Falla, T. J. *Clin. Dermatol.* **2009**, *27*, 485–494.
93. Li, S. S.; Tsai, H. J. *Fish Shellfish Immunol.* **2009**, *26*, 316–325.
94. Pitta, M. G. D.; Pitta, M. G. D.; Galdino, S. L. *Curr. Protein Pept. Sci.* **2010**, *11*, 236–247.
95. Ireland, D. C.; Wang, C. K. L.; Wilson, J. A.; Gustafson, K. R.; Craik, D. J. *Biopolymers* **2008**, *90*, 51–60.
96. Berrocal-Lobo, M.; Molina, A.; Rodriguez-Palenzuela, P.; Garcia-Medo, F.; Rivas, L. *Exp. Parasitol.* **2009**, *122*, 247–249.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

97. López-García, B.; Veyrat, A.; Pérez-Payá, E.; González-Candelas, L.; Marcos, J. F. *Int. J. Food Microbiol.* **2003**, *89*, 163–170.
98. Janisiewicz, W. J.; Pereira, I. B.; Almeida, M. S.; Roberts, D. P.; Wisniewski, A.; Kurtenbach, E. *Postharvest Biol. Technol.* **2008**, *47*, 218–225.
99. Jones, R. W.; Prusky, D. *Phytopathology* **2002**, *92*, 33–37.
100. Epple, P.; Apel, K.; Bohlmann, H. *Plant Cell* **1997**, *9*, 509–520.
101. De Coninck, B. M. A.; Sels, J.; Venmans, E.; Thys, W.; Goderis, I. J. W. M.; Carron, D.; Delaure, S. L.; Cammue, B. P. A.; De Bolle, M. F. C.; Mathys, J. *New Phytol.* **2010**, *187*, 1075–1088.
102. Kostov, K.; Christova, P.; Slavov, S.; Batchvarova, R. *Biotechnol. Biotechnol. Equip.* **2009**, *23*, 1121–1125.
103. Jha, S.; Chattoo, B. B. *Transgenic Res.* **2010**, *19*, 373–384.
104. Li, Z.; Zhou, M. P.; Zhang, Z. Y.; Ren, L. J.; Du, L. P.; Zhang, B. Q.; Xu, H. J.; Xin, Z. Y. *Funct. Integr. Genom.* **2011**, *11*, 63–70.
105. Choi, M. S.; Kim, Y. H.; Park, H. M.; Seo, B. Y.; Jung, J. K.; Kim, S. T.; Kim, M. C.; Shin, D. B.; Yun, H. T.; Choi, I. S.; Kim, C. K.; Lee, J. Y. *Mol. Cells* **2009**, *28*, 131–137.
106. Gao, A. G.; Hakimi, S. M.; Mittanck, C. A.; Wu, Y.; Woerner, B. M.; Stark, D. M.; Shah, D. M.; Liang, J. H.; Rommens, C. M. T. *Nat. Biotechnol.* **2000**, *18*, 1307–1310.
107. Carmona, M. J.; Molina, A.; Fernandez, J. A.; Lopezfando, J. J.; Garciaolmedo, F. *Plant J.* **1993**, *3*, 457–462.
108. Chan, Y. L.; Prasad, V.; Sanjaya; Chen, K.; Liu, P.; Chan, M.; Cheng, C. *Planta* **2005**, *221*, 386–393.
109. Iwai, T.; Kaku, H.; Honkura, R.; Nakamura, S.; Ochiai, H.; Sasaki, T.; Ohashi, Y. *Mol. Plant-Microbe Interact.* **2002**, *15*, 515–521.
110. Zhu, Y. J.; Agbayani, R.; Moore, P. H. *Planta* **2007**, *226*, 87–97.
111. Jha, S.; Tank, H. G.; Prasad, B. D.; Chattoo, B. B. *Transgenic Res.* **2009**, *18*, 59–69.
112. Turrini, A.; Sbrana, C.; Pitto, L.; Castiglione, M. R.; Giorgetti, L.; Briganti, R.; Bracci, T.; Evangelista, M.; Nuti, M. P.; Giovannetti, M. *New Phytol.* **2004**, *163*, 393–403.
113. Kanzaki, H.; Nirasawa, S.; Saitoh, H.; Ito, M.; Nishihara, M.; Terauchi, R.; Nakamura, I. *Theor. Appl. Genet.* **2002**, *105*, 809–814.
114. Ntui, V. O.; Thirukkumaran, G.; Azadi, P.; Khan, R. S.; Nakamura, I.; Mii, M. *Plant Cell Rep.* **2010**, *29*, 943–954.
115. Anuradha, T. S.; Divya, K.; Jami, S. K.; Kirti, P. B. *Plant Cell Rep.* **2008**, *27*, 1777–1786.
116. Portieles, R.; Ayra, C.; Gonzalez, E.; Gallo, A.; Rodriguez, R.; Chacon, O.; Lopez, Y.; Rodriguez, M.; Castillo, J.; Pujol, M.; Enriquez, G.; Borroto, C.; Trujillo, L.; Thomma, B. P. H. J.; Borras-Hidalgo, O. *Plant Biotechnol. J.* **2010**, *8*, 678–690.
117. Schaefer, S. C.; Gasic, K.; Cammue, B.; Broekaert, W.; van Damme, E. J. M.; Peumans, W. J.; Korban, S. S. *Planta* **2005**, *222*, 858–866.
118. Prasad, B. D.; Jha, S.; Chattoo, B. B. *Plant Sci.* **2008**, *175*, 364–371.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

119. Koo, J. C.; Chun, H. J.; Park, H. C.; Kim, M. C.; Koo, Y. D.; Koo, S. C.; Ok, H. M.; Park, S. J.; Lee, S. H.; Yun, D. J.; Lim, C. O.; Bahk, J. D.; Lee, S. Y.; Cho, M. J. *Plant Mol. Biol.* **2002**, *50*, 441–452.
120. Lee, O. S.; Lee, B.; Park, N.; Koo, J. C.; Kim, Y. H.; Prasad, T.; Karigar, C.; Chun, H. J.; Jeong, B. R.; Kim, D. H.; Nam, J.; Yun, J. G.; Kwak, S. S.; Cho, M. J.; Yun, D. J. *Phytochemistry* **2003**, *62*, 1073–1079.
121. Liang, H. Y.; Catranis, C. M.; Maynard, C. A.; Powell, W. A. *Biotechnol. Lett.* **2002**, *24*, 383–389.
122. Kazan, K.; Rusu, A.; Marcus, J. P.; Goulter, K. C.; Manners, J. M. *Mol. Breed.* **2002**, *10*, 63–70.
123. Krishnamurthy, K.; Balconi, C.; Sherwood, J. E.; Giroux, M. J. *Mol. Plant-Microbe Interact.* **2001**, *14*, 1255–1260.
124. Zhang, J. R.; Martin, J. M.; Balint-Kurti, P.; Huang, L.; Giroux, M. J. *J. Phytopathol.* **2011**, *159*, 188–190.
125. Molina, A.; García-Olmedo, F. *Plant J.* **1997**, *12*, 669–675.
126. Roy-Barman, S.; Sautter, C.; Chattoo, B. B. *Transgenic Res.* **2006**, *15*, 435–446.
127. Li, X. Q.; Gasic, K.; Cammue, B.; Broekaert, W.; Korban, S. S. *Planta* **2003**, *218*, 226–232.
128. Bi, Y. M.; Cammue, B. P. A.; Goodwin, P. H.; KrishnaRaj, S.; Saxena, P. K. *Plant Cell Rep.* **1999**, *18*, 835–840.
129. Patkar, R. N.; Chattoo, B. B. *Mol. Breed.* **2006**, *17*, 159–171.
130. Almasia, N. I.; Bazzini, A. A.; Hopp, H. E.; Vazquez-Rovere, C. *Mol. Plant Pathol.* **2008**, *9*, 329–338.
131. Balaji V.; Smart C. D. *Transgenic Res.* **2011,** DOI 10.1007/s11248-011-9506-x.
132. Cavallarin, L.; Andreu, D.; San Segundo, B. *Mol. Plant-Microbe Interact.* **1998**, *11*, 218–227.
133. Sharma, A.; Sharma, R.; Imamura, M.; Yamakawa, M.; Machii, H. *FEBS Lett.* **2000**, *484*, 7–11.
134. Florack, D.; Allefs, S.; Bollen, R.; Bosch, D.; Visser, B.; Stiekema, W. *Transgenic Res.* **1995**, *4*, 132–141.
135. Mills, D.; Hammerschlag, F. A.; Nordeen, R. O.; Owens, L. D. *Plant Sci.* **1994**, *104*, 17–22.
136. Owens, L. D.; Heutte, T. M. *Mol. Plant-Microbe Interact.* **1997**, *10*, 525–528.
137. Coca, M.; Peñas, G.; Gómez, J.; Campo, S.; Bortolotti, C.; Messeguer, J.; San Segundo, B. *Planta* **2006**, *223*, 392–406.
138. Ko, K. S.; Norelli, J. L.; Reynoird, J. P.; Aldwinckle, H. S.; Brown, S. K. *J. Am. Soc. Hort. Sci.* **2002**, *127*, 515–519.
139. Borejsza-Wysocka, E.; Norelli, J. L.; Aldwinckle, H. S.; Malnoy, M. *BMC Biotechnol.* **2010**, *10*.
140. Vila, L.; Lacadena, V.; Fontanet, P.; Martínez del Pozo, A.; San Segundo, B. *Mol. Plant-Microbe Interact.* **2001**, *14*, 1327–1331.
141. Coca, M.; Bortolotti, C.; Rufat, M.; Peñas, G.; Eritja, R.; Tharreau, D.; Martínez del Pozo, A.; Messeguer, J.; San Segundo, B. *Plant Mol. Biol.* **2004**, *54*, 245–259.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

142. Aerts, A. M.; Thevissen, K.; Bresseleers, S. M.; Sels, J.; Wouters, P.; Cammue, B. P. A.; François, I. E. J. A. *Plant Cell Rep.* **2007**, *26*, 1391–1398.
143. Zakharchenko, N. S.; Rukavtsova, E. B.; Gudkov, A. T.; Buryanov, Y. I. *Russ. J. Gen.* **2005**, *41*, 1187–1193.
144. Jan, P. S.; Huang, H. Y.; Chen, H. M. *Appl. Environ. Microbiol.* **2010**, *76*, 769–775.
145. Cardoso, S. C.; Barbosa-Mendes, J. M.; Boscariol-Camargo, R. L.; Christiano, R. S. C.; Bergamin, A.; Vieira, M. L. C.; Mendes, B. M. J.; Mourao, F. D. A. *Plant Mol. Biol. Rep.* **2010**, *28*, 185–192.
146. Rahnamaeian, M.; Langen, G.; Imani, J.; Khalifa, W.; Altincicek, B.; von Wettstein, D.; Kogel, K. H.; Vilcinskas, A. *J. Exp. Bot.* **2009**, *60*, 4105–4114.
147. Banzet, N.; Latorse, M. P.; Bulet, P.; François, E.; Derpierre, C.; Dubald, M. *Plant Sci.* **2002**, *162*, 995–1006.
148. Langen, G.; Imani, J.; Altincicek, B.; Kieseritzky, G.; Kogel, K. H.; Vilcinskas, A. *Biol. Chem.* **2006**, *387*, 549–557.
149. Ohshima, M.; Mitsuhara, I.; Okamoto, M.; Sawano, S.; Nishiyama, K.; Kaku, H.; Natori, S.; Ohashi, Y. *J. Biochem.* **1999**, *125*, 431–435.
150. Imamura, T.; Yasuda, M.; Kusano, H.; Nakashita, H.; Ohno, Y.; Kamakura, T.; Taguchi, S.; Shimada, H. *Transgenic Res.* **2010**, *19*, 415–424.
151. Allefs, S. J. H. M.; Dejong, E. R.; Florack, D. E. A.; Hoogendoorn, C.; Stiekema, W. J. *Mol. Breeding* **1996**, *2*, 97–105.
152. Ponti, D.; Mangoni, M. L.; Mignogna, G.; Simmaco, M.; Barra, D. *Biochem. J.* **2003**, *370*, 121–127.
153. Yevtushenko, D. P.; Misra, S. *Plant Biotechnol. J.* **2007**, *5*, 720–734.
154. Arenas, G.; Marshall, S. H.; Espinoza, V.; Ramirez, I.; Pena-Cortes, H. *Electron. J. Biotechnol.* **2006**, *9*, 144–151.
155. Moreno, A. B.; Peñas, G.; Rufat, M.; Bravo, J. M.; Estopà, M.; Messeguer, J.; San Segundo, B. *Mol. Plant-Microbe Interact.* **2005**, *18*, 960–972.
156. Oldach, K. H.; Becker, D.; Lorz, H. *Mol. Plant-Microbe Interact.* **2001**, *14*, 832–838.
157. Jaynes, J. M.; Nagpala, P.; Destefanobeltran, L.; Huang, J. H.; Kim, J. H.; Denny, T.; Cetiner, S. *Plant Sci.* **1993**, *89*, 43–53.
158. Jones, R. W.; Ospina-Giraldo, M. D.; Clemente, T. *Mol. Breeding* **2004**, *14*, 83–89.
159. Yevtushenko, D. P.; Romero, R.; Forward, B. S.; Hancock, R. E. W.; Kay, W. W.; Misra, S. *J. Exp. Bot.* **2005**, *56*, 1685–1695.
160. Osusky, M.; Zhou, G.; Osuska, L.; Hancock, R. E. W.; Kay, W. W.; Misra, S. *Nat. Biotechnol.* **2000**, *18*, 1162–1166.
161. Osusky, M.; Osuska, L.; Kay, W.; Misra, S. *Theor. Appl. Genet.* **2005**, *111*, 711–722.
162. Osusky, M.; Osuska, L.; Hancock, R. E. W.; Kay, W. W.; Misra, S. *Transgenic Res.* **2004**, *13*, 181–190.
163. Cary, J. W.; Rajasekaran, K.; Jaynes, J. M.; Cleveland, T. E. *Plant Sci.* **2000**, *154*, 171–181.
164. Mentag, R.; Luckevich, M.; Morency, M. J.; Seguin, A. *Tree Physiol.* **2003**, *23*, 405–411.

165. Rajasekaran, K.; Cary, J. W.; Jaynes, J. M.; Cleveland, T. E. *Plant Biotechnol. J.* **2005**, *3*, 545–554.

166. Chakrabarti, A.; Ganapathi, T. R.; Mukherjee, P. K.; Bapat, V. A. *Planta* **2003**, *216*, 587–596.

167. DeGray, G.; Rajasekaran, K.; Smith, F.; Sanford, J.; Daniell, H. *Plant Physiol.* **2001**, *127*, 852–862.

168. Vidal, J. R.; Kikkert, J. R.; Malnoy, M. A.; Wallace, P. G.; Barnard, J.; Reisch, B. I. *Transgenic Res.* **2006**, *15*, 69–82.

169. Alan, A. R.; Blowers, A.; Earle, E. D. *Plant Cell Rep.* **2004**, *22*, 388–396.

170. Li, Q. S.; Lawrence, C. B.; Xing, H. Y.; Babbitt, R. A.; Bass, W. T.; Maiti, I. B.; Everett, N. P. *Planta* **2001**, *212*, 635–639.

171. Xing, H. Y.; Lawrence, C. B.; Chambers, O.; Davies, H. M.; Everett, N. P.; Li, Q. Q. *Planta* **2006**, *223*, 1024–1032.

172. Bhargava, A.; Osusky, M.; Hancock, R. E. W.; Forward, B. S.; Kay, W. W.; Misra, S. *Plant Sci.* **2007**, *172*, 515–523.

173. Bhargava, A.; Osusky, M.; Forward, B. S.; Hancock, R. E. W.; Kay, W. W.; Misra, S. *Plant Cell Tissue Organ Cult.* **2007**, *88*, 301–312.

174. Liu, B.; Hibbard, J. K.; Urwin, P. E.; Atkinson, H. J. *Plant Biotechnol. J.* **2005**, *3*, 487–496.

175. Newhouse, A. E.; Schrodt, F.; Liang, H. Y.; Maynard, C. A.; Powell, W. A. *Plant Cell Rep.* **2007**, *26*, 977–987.

176. Campo, S.; Manrique, S.; Garcia-Martinez, J.; Segundo, B. S. *Plant Biotechnol. J.* **2008**, *6*, 585–608.

177. Capparelli, R.; Palumbo, D.; Iannaccone, M.; Ventimiglia, I.; Di Salle, E.; Capuano, F.; Salvatore, P.; Amoroso, M. *Biotechnol. Lett.* **2006**, *28*, 943–949.

178. Wu, Y.; He, Y.; Ge, X. C. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1303–1310.

179. Macauley-Patrick, S.; Fazenda, M. L.; McNeil, B.; Harvey, L. M. *Yeast* **2005**, *22*, 249–270.

180. López-García, B.; Moreno, A. B.; Sansegundo, B.; de los Rios, V.; Manning, J. M.; Gavilanes, J. G.; Martínez del Pozo, A. *Protein Expression Purif.* **2010**, *70*, 206–210.

181. Boothe, J.; Nykiforuk, C.; Shen, Y.; Zaplachinski, S.; Szarka, S.; Kuhlman, P.; Murray, E.; Morck, D.; Moloney, M. M. *Plant Biotechnol. J.* **2010**, *8*, 588–606.

182. Oey, M.; Lohse, M.; Scharff, L. B.; Kreikemeyer, B.; Bock, R. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 6579–6584.

183. Lee, S. B.; Li, B. C.; Jin, S. X.; Daniell, H. *Plant Biotechnol. J.* **2011**, *9*, 100–115.

184. Dornenburg, H. *Biotechnol. Lett.* **2008**, *30*, 1311–1321.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch013

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Chapter 14

Strategies for Controlling Plant Diseases and Mycotoxin Contamination Using Antimicrobial Synthetic Peptides

K. Rajasekaran,*,[1] J. W. Cary,[1] C. A. Chlan,[2] J. M. Jaynes,[3] and D. Bhatnagar[1]

[1]Food and Feed Safety Research Unit, Southern Regional Research Center, Agricultural Research Service, USDA, New Orleans, LA 70124
[2]Biology Department, University of Louisiana at Lafayette, Lafayette, LA 70504
[3]Integrative Biosciences, Tuskegee University, Tuskegee, AL 36811
***E-mail: Rajah.Rajasekaran@ars.usda.gov. Phone: 504-286-4482. Fax: 504-286-4533**

Development of disease-resistant transgenic crops is made difficult because host plant-pathogen interactions are complex and often crop/variety or pathogen/strain-specific. Synthetic peptides are useful in controlling a broad-spectrum of plant pathogens including the difficult-to-control, mycotoxin-producing fungal species such as *Aspergillus* and *Fusarium*. The effectiveness of synthetic peptide genes in transgenic crops for controlling microbial pathogens has been well established in our laboratory and elsewhere. Some of the additional advantages of synthetic peptides include 1) they are not subject to rapid degradation in cytoplasm as compared to naturally occurring peptides, 2) they offer unique resistance to microbes because of their novelty and preempt possible development of resistance, and 3) preliminary studies indicate that they are not harmful to beneficial microbes in the rhizosphere. This paper reviews various strategies of synthetic peptide gene expression in transgenic crops for controlling or inhibiting plant pathogens including mycotoxin producing fungi.

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Keywords: aflatoxin; biotechnology; disease resistance; food and feed safety; mycotoxin; phytopathogens; synthetic peptide; transgenic expression

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Introduction

Development of disease-resistant transgenic crops is made difficult because host plant-pathogen interactions are complex and often crop/variety or pathogen/strain-specific. In addition, microbial pathogens can develop resistance to plant defense proteins and peptides which quite often are unstable, lack specificity and may be toxic to non-target species. There are excellent reviews in this book and elsewhere of the different types of naturally-occurring antimicrobial peptides (AMPs) (*1–4*). Novel synthetic peptides have been developed to overcome many of the deficiencies characteristic of natural AMPs. Recent advances in combinatorial chemistry and automated peptide synthesis have paved the way for rational design of stable, potent, and novel synthetic peptides with target-specific biological activity. Some of these lytic, synthetic peptides have already been expressed in transgenic plants with varying degrees of success against fungal and bacterial plant pathogens (*1*, *2*). In addition to controlling crop losses due to microbial pathogens, our laboratory is interested in controlling or eliminating some fungal pathogens that cause contamination of food and feed crops with mycotoxins. Our primary target is *Aspergillus* species which can produce aflatoxins when they contaminate several oil-rich seed crops such as cottonseed, corn, peanut and tree nuts including pistachio, almonds, pecans, walnut and Brazil nut.

Aflatoxins are toxic, highly carcinogenic secondary metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus*, during fungal infection of a susceptible crop in the field or after harvest. Highly reactive aflatoxin derivatives (8, 9-epoxy-aflatoxin B_1) can intercalate DNA and form DNA-aflatoxin adducts. This results in cellular repair of the adducts often leading to G to T transversions and subsequent mutations in the coding region of genes, particularly the tumor suppressor gene, p53 (*5*) from patients exposed to high dietary aflatoxin B_1 (AFB_1) (*6*, *7*). A very high incidence (67%) of liver carcinomas in Senegal, China, Swaziland and Mozambique bears the characteristics of aflatoxin-induced mutation of the p53 tumor suppressor gene. This mutation has also been associated with liver cancer in Mexico. The binding of AFB_1 to DNA also leads to the formation of single-stranded gaps. As a result, it inhibits DNA polymerase activity at DNA binding sites. This stimulates an error-prone repair system that may induce mutations.

Human exposure to aflatoxins can result directly from ingestion of contaminated foods, or indirectly from consumption of foods from animals previously exposed to aflatoxins in feeds (*8*). Human aflatoxicosis continues to be an occasional, serious problem. For example, a severe outbreak of acute hepatotoxicity was reported in Kenya in 2002 (*9*). Half of the maize food samples tested in districts associated with this outbreak had AFB1 levels >20 ppb (the action level for AFB_1 in Kenya), with 3% to 12% of samples, depending on the

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

district, containing >1000 ppb and some samples containing as much as 8000 ppb AFB_1. This outbreak had at least a 39% incidence of death (317 cases with 125 deaths) (*10*). Foodstuffs contaminated with aflatoxins have also been associated with increased incidence of liver cancer in humans (*11*). The main target organ of aflatoxins is the liver. Typical symptoms of aflatoxicosis in animals include proliferation of the bile duct, centrilobular necrosis and fatty infiltration of the liver, generalized hepatic lesions and hepatomas (*7*, *12*). AFB_1 also affects other organs and tissues including the lungs and the entire respiratory system. Ingestion of aflatoxin-contaminated feed by farm animals leads to substantial loss of productivity and degradation of meat quality (*13*). The susceptibility of animals to AFB_1 varies considerably with species in the following order: rabbits (most), ducklings, mink, cats, pigs, trout, dogs, guinea pigs, sheep, monkeys, chickens, rats, mice, and hamsters (least). Regarding the carcinogenic effects, rats, rainbow trout, monkeys, and ducks are most susceptible and mice are relatively resistant (*14*).

A recent study (*15*) revealed a strong association between exposure to aflatoxin in West African children and stunted growth (a reflection of chronic malnutrition) and low body weight (a reflection of acute malnutrition). Therefore, aflatoxin contamination of food and feed not only significantly reduces the value of grains but also poses serious health and nutritional threats to human and farm animals (*16–18*).

The problem of aflatoxin contamination of food and feed exists in many countries, especially in tropical and subtropical regions where conditions of temperature and humidity are optimal for growth of the fungi and for production of the toxin (*8*, *19*). Vardon *et al.* (*20*) estimated that nationwide annual loss of crop revenues due to aflatoxin and other mycotoxin contamination in the United States ranged from $418 million to 1.66 billion, with the mean about $932 million. However, estimates for a single year do not provide a true picture of the extent of aflatoxin contamination because of variability in contamination levels in different years. For cottonseed, the Arizona Cotton Research and Protection Council estimated the loss of revenues due to aflatoxin contamination in Arizona alone during a 22-year period from 1977 to 1999 to be an average of $4.4 million per year (*21*). In one year (1999), the loss of value to South Texas cotton producers was over $7 million. For corn, the direct cost of aflatoxin contamination during the 1980s to all the southern states was estimated to be greater than $237 million (*22*); whereas losses in a single year (1998) in Mississippi, Louisiana, and Texas were extremely severe, and valued at over $17 million (*21*). Therefore, eliminating aflatoxin from food and feedstuffs to enhance food safety and security is a major global effort.

The pre-harvest control of aflatoxin contamination, which is the main area of our current research and the focus of this chapter, includes strategies such as good cultural practices, biocontrol using competing microbes, understanding of host resistance mechanisms and enhancing host resistance through breeding and genetic engineering. Post-harvest control strategies include adequate storage and monitoring as well as physical chemical or biological detoxification of contaminated food and feed. These topics have been adequately covered in several other reviews (*14*, *19*, *23–26*).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Genesis of Synthetic Peptides

Lytic peptides are small proteins that are major components of the antimicrobial defense systems of numerous species (*27*, *28*). They are a ubiquitous feature of nearly all multi-cellular and some single-cellular life forms. They generally consist of between 10-40 amino acid sequences, which have potential for forming discrete secondary structures. Often, they exhibit the property of amphipathy. An amphipathic α-helix may be depicted as a cylinder with one curved face composed primarily of nonpolar amino acids while the other face is composed of polar amino acids.

In early 1990s, Jaynes and his collaborators utilized the gene encoding a close homolog of cecropin B (SB-37) to augment bacterial disease resistance in plants. However, during the course of their studies, a new highly sequence divergent peptide was synthesized (Shiva-1) and was shown to possess a more potent lytic activity than SB-37 (*29*, *30*). The enhanced bioactivity of Shiva-1 was the first indication that modifications made in the primary sequence of lytic peptides would not destroy the peptide's activity provided certain physical characteristics of the peptide were conserved. Indeed, this was a paradigm-shifting moment in understanding of the structure/function relationship of these incredibly interesting natural molecules and paved the way for the design of novel molecules with enhanced activities. Similarly, Coca and her colleagues (*31*) reported that two plant codon optimized synthetic cecropin A genes, which were designed either to retain the cecropin A peptide in the endoplasmic reticulum, or to secrete cecropin A to the extracellular space, were expressed in transgenic rice. The inhibitory activity of protein extracts prepared from leaves of cecropin A-expressing plants on the *in vitro* growth of *Magnaporthe grisea,* the causal agent of the rice blast disease, indicated that the cecropin A protein produced by the transgenic rice plants was biologically active against rice blast at various levels. Another example of sequence modification resulting in increased potency of natural peptides is provided in MSI-99, a synthetic analog of magainin-II that displayed more positive charge and antibacterial and antifungal activity than its predecessor (*32*, *33*).

Most of the α-helical lytic peptides that have been described in the literature fall into one of three different classes based on the arrangement of amphipathy and high positive charge density within the molecule (*2*):

1) Cecropins (35 amino acids in length and derived from the Giant Silk Moth), N-terminal half amphipathic while the C-terminal half mostly hydrophobic (*34*);
2) Magainins (23 amino acids in length and derived from the African Clawed Frog), amphipathic the full-length of the molecule (*35*); and
3) Melittin (26 amino acids in length and derived from the Honeybee), C-terminal half amphipathic with the N-terminal half primarily hydrophobic (*36*).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

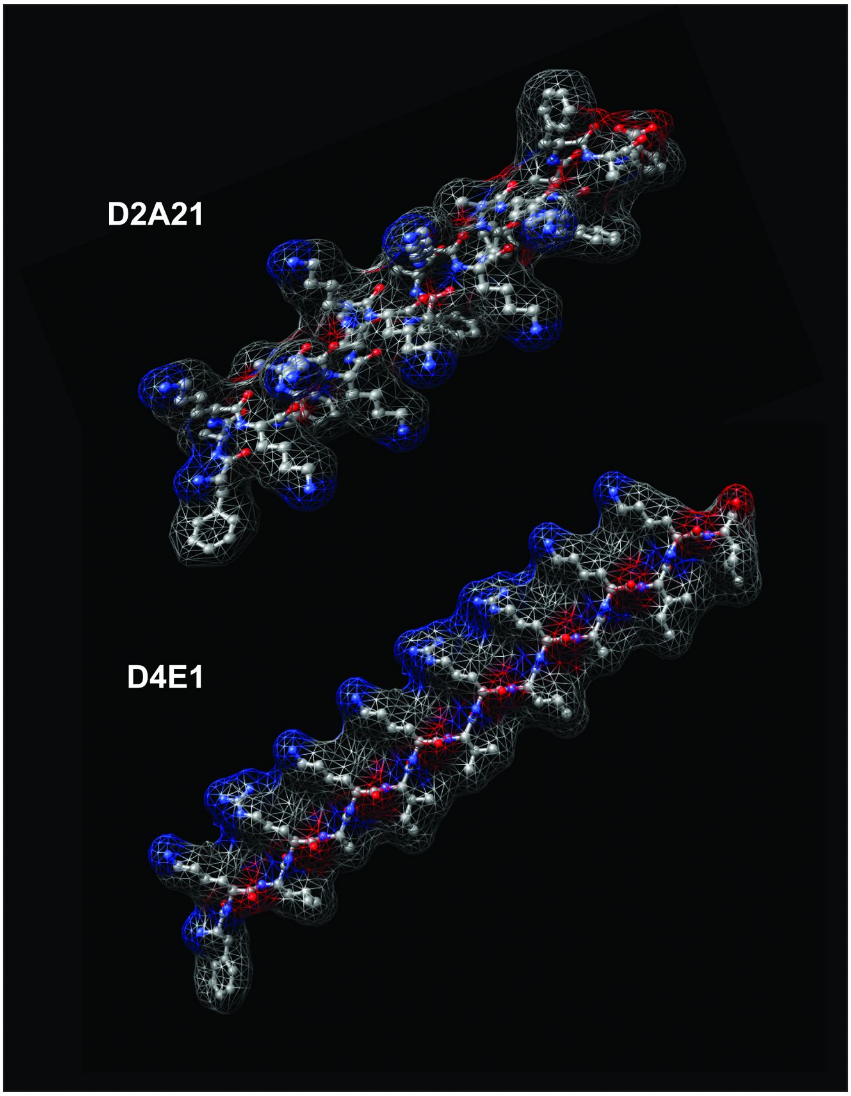


Figure 1. Structural representation of D2A21 (α-helical) and D4E1 (β-sheet) peptides. (see color insert)

β-sheet peptides include defensins and protegrins. These peptides can assume this shape because of intra-disulfide linkages that lock them into this form, an absolute requisite for activity. We have designed a novel class of peptides that form β-sheets without the necessity of disulfide linkages. Structural designs of an α-helical (D2A21) and a β-sheet (D4E1) peptides are provided in Figure 1.

a. Physical Properties That Unify Antimicrobial Peptide Structure and Function: Amphipathy, Hydrophobicity, and Charge Density

There are a number of physical features that play a role in modulating the activity of these types of peptides including degree and length of amphipathy, hydrophobicity, surface area of both hydrophilic and hydrophobic faces, length and hydrophobicity of amphipathic section, heterogeneity and placement of amphipathic section (N or C terminal), "+" charge density, presence, length and hydrophobicity of tail, predominating secondary structure and steric or volume considerations (*2*). The differences in their physical attributes of amino acids, the building blocks of proteins, provide ample opportunities to design their antimicrobial characteristics.

To facilitate exploration of the plasticity of the structure/function paradigm of designed AMPs, a simple method was devised more than 20 years ago (*37*, *38*) to illustrate the physical relationships among AMPs. In this method, peptide sequences are displayed so that their structural differences and similarities are readily visualized. The method uses the font called "Molly" (*2*). Molly has been helpful in delineating the clues to lytic peptide structure/function. It also allows for an analysis of other unknown, functionally uncharacterized proteins to aid in pattern recognition and correlation of protein function of structurally distinct sequences of related and unrelated proteins.

Naturally occurring and designed AMPs can and do differ significantly in amino acid sequence, but they retain a characteristically positively charged and potentially amphipathic alpha-helical or beta-pleated sheet structure (*37*, *38*), indicating that the specific amino acid sequence of the AMP is irrelevant to peptide function as long as certain physical properties of the peptide are maintained. Design and large scale production of synthetic peptides (*39*) with relevance to antimicrobial activity is further explored in one of the chapters in this book (*121*).

b. Antimicrobial Activity of Synthetic Peptides

Antimicrobial activities of synthetic peptides have been determined in our laboratory and others through bioassays of fungal and bacterial pathogens. The inhibitory effects of two synthetic peptides (D4E1 and AGM184 based on tachyplesin) on pre-germinated spores of *Aspergillus flavus* are provided in Figure 2.

A partial list of microbial pathogens controlled by some of the synthetic peptides (D4E1, D2A21, D5C, MSI-99), either added to culture-media and/or expressed in transgenic plants is given in Table 1 (*29*, *33*, *40–47*).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

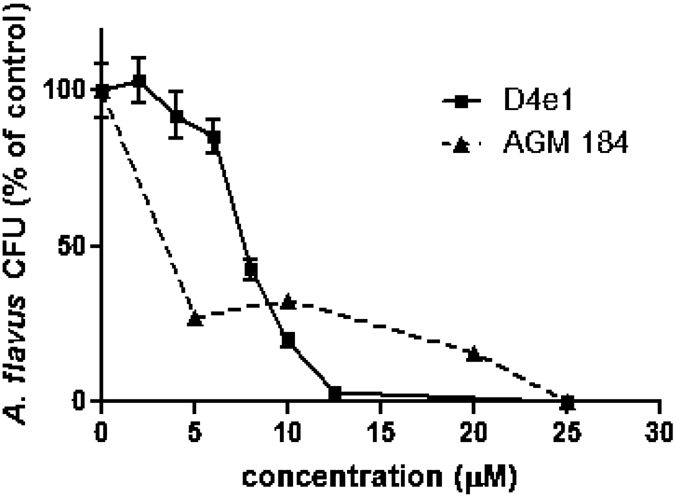


Figure 2. Effect of D4E1 and AGM 184 (tachyplesin-based) on Aspergillus flavus.

c. Mode of Action of Synthetic Peptides

While there is a significant volume of information available on the mode of action of natural, cationic antimicrobial peptides (AMPs) on bacteria and to a lesser extent fungi (*48*–*52*), less is known about the mode of action of peptide analogs and synthetic peptides against fungi, especially those filamentous fungi associated with plant disease. Very few cationic AMPs have been extensively studied and there remains a significant level of uncertainty as to specific mechanisms of action. What is known is that the central dogma of cell death via membrane disruption cannot entirely explain the observed activity of the multitude of AMPs found in nature. Studies suggest that AMPS can be classified as membrane-disruptive or as membrane non-disruptive with respect to their modes of action (*48*). The membrane disruptive model involves a discrimination mechanism whereby cationic AMPs that target the cytoplasmic membrane can specifically target either prokaryotic or eukaryotic membranes (*53*).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Table 1. List of fungal and bacterial pathogens controlled by synthetic peptides (*references in parentheses*)

Fungal pathogens			
Alternaria spp.	(*42*, *72*, *73*)	*Gremmeniella abietina*	(*43*, *44*)
Alternaria alternata	(*42*, *70*)	*Melampsora medusa*	(*43*, *44*)
Alternaria solani	(*42*)(*68*)(*71*)	*Mycosphaerella musicola*	(*70*)
Aspergillus flavus	(*33*, *41*, *42*, *66*)	*Nectria galligena*	(*43*, *44*)
Aspergillus flavus 70-GFP	(*42*, *64*, *66*)	*Ophistoma ulmi*	(*43*, *44*)
Aspergillus fumigatus	(*55*)	*Penicillium italicum*	(*42*)
Aspergillus niger	(*47*)	*Phytophthora capsici*	(*122*)
Botrytis cinerea	(*70*)	*Phytophthora cinnamomi*	(*42*)
Cercospora spp.	(*72*, *73*)	*Phytophthora infestans*	(*71*)
Cercospora kikuchii	(*42*)	*Phytophthora palmivora*	(*122*)
Claviceps purpurea	(*42*)	*Phytophthora parasitica*	(*42*)
Colletotrichum destructivum	(*33*, *41*, *42*)	*Pythium ultimum*	(*42*)
Cronartium ribicola	(*43*, *44*)	*Rhizoctonia solani*	(*42*, *72*, *73*)
Fusarium spp	(*72*, *73*)	*Sclerotinia minor*	(*45*)
Fusarium graminearum	(*42*)	*Sclerotium rolfsii*	(*45*)
Fusarium oxysporum	(*42*, *55*)	*Sclerotinia sclerotiorum*	(*70*)
Fusarium oxysporum f.sp. *cubense*	(*70*)	*Septoria musiva*	(*43*, *44*)
Fusarium oxysporum f.sp. *vasinfectum*	(*63*, *67*)	*Thielaviopsis basicola*	(*42*, *64*)
*Fusarium verticillioides (*syn. *F. moniliforme)*	(*33*, *41*, *42*, *55*, *64*)	*Verticillium dahliae*	(*33*, *41*, *42*, *64*, *72*, *73*)
Bacterial pathogens			
Agrobacterium tumefaciens	(*46*)	*Pseudomonas syringae* pv. *tabaci*	(*33*, *42*, *65*)
Erwinia amylovora	(*69*)	*Pseudomonas syringae pv. tomato*	(*32*, *71*)
*Pectobacterium carotovorum (*syn. *Erwinia carotovora)*	(*72*, *73*, *90*)	*Xanthomonas campestris* pv. *malvacearum*	(*42*)

Continued on next page.

Table 1. (Continued). List of fungal and bacterial pathogens controlled by synthetic peptides (*references in parentheses*)

Bacterial pathogens			
Pseudomonas spp.	(*33*)	*Xanthomonas populi* pv. *populi*	(*46*)
Pseudomonas solanacearum	(*29*)		

The specificity toward membranes of different classes of organisms is based on the differences in the lipid composition of the membranes with prokaryotes having mostly negatively charged phospholipids and eukaryotes having mainly zwitterionic phospholipids with the lipid in the latter being cholesterol (vertebrates)/ergosterol (fungi) and sphingomyelin (animals). In eukaryotes, membrane permeabilization by AMPs occurs via insertion of AMP molecules into the membrane and subsequent formation of an aqueous pore (barrel-stave mechanism). In prokaryotic cells the AMPs tend to align parallel to the membrane and interact with the negatively charged head groups of the phospholipid eventually forming micelles that lead to membrane disintegration (carpet mechanism) (*54*). Membrane permeabilization appears to be one of the main mechanisms by which the synthetic, amphipathic, peptides D4E1 and D2A21 were found to inhibit the growth of germinating spores of the mycotoxigenic filamentous fungus, *A. flavus*, as well as a number of other agriculturally important phytopathogens (*2*, *43*). Microscopic analysis of germinating conidia treated with the peptides showed that plasma membranes as well as nuclear and mitochondrial envelopes were difficult to discern or were distorted and swollen (Figure 3). Physiochemical studies of the antifungal peptide D4E1 demonstrated that D4E1 complexed with ergosterol present in the conidial cell walls (*55*).

More information is coming to light in support of the non-disruptive mode of action that suggests certain AMPs have the ability to translocate across cell membranes (*1*, *48*, *56*). Once internalized, antimicrobial activity is dependent in large part on interaction of the AMP with internal targets or in synergy with membrane disruptive mechanisms. Intracellular targets of AMPs can include anionic molecules such as DNA and RNA that can lead to inhibition of DNA/protein synthesis, inhibition of enzymes involved in synthesis of cell wall components as well as the induction of toxic reactive oxygen species (ROS) and apoptosis (*56–59*). Studies on the antifungal peptides *Rs*-AFP2, melittin and psacotheasin have shown that in addition to disrupting plasma membranes of *Candida albicans*, these peptides also elicit generation of ROS that in turn may play a key role in the induction of programmed cell death (apoptosis) (*57*, *60*, *61*). Uptake of antifungal peptides has been demonstrated in filamentous fungi. The pea defensin, Psd1, was shown to enter the hyphae of *Neurospora crassa* and enter the nucleus where it interacts with the cell cycle control protein cyclin F, halting the cell cycle, thus leading to death (*62*). Uptake of the tobacco defensin, NaD1 was also demonstrated in the agronomically important filamentous fungus, *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) (*63*). A model was proposed by

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

which NaD1 binds to the Fov cell wall resulting in rapid permeabilization of the plasma membrane to allow entry of NaD1 into the hyphae. It was then proposed that NaD1 interacts with intracellular targets that alone or in combination with cell membrane leakage induce programmed cell death possibly by a ROS-induced apoptotic-like mechanism. Though the exact mechanisms by which natural or synthetic AMPs inhibit the growth of plant pathogenic fungi is not well understood at this time, studies on other filamentous and yeast-like fungi indicate that cell permeabilization may be necessary but not sufficient to bring about cell death. In addition to damaging cellular membranes, certain AMPs may disrupt fungal signal-transduction cascades and thereby activate stress-responsive signal-transduction pathways in the fungus which, in turn, activate apoptotic pathways leading to death of the invading fungus. In the case of transgenic plants expressing AMPs it will be necessary not only to analyze the effects of the AMP on the invading fungal pathogen *in planta,* but also to understand what effects, if any, that the AMP may have on the host plant's cells. It may be that in addition to activating fungal apoptotic pathways the AMP is indirectly inhibiting fungal growth via activation of host defense response pathways that may or may not be deleterious to the host plant.

Nuclear Expression of Synthetic Peptides in Transgenic Plants

There are several reports on the effective use of synthetic peptides towards control of plant pathogens; both fungal and bacterial (Table 1). In our laboratory, we demonstrated the broad-spectrum activity of a β-sheet linear synthetic peptide, D4E1 (*41*, *42*, *64*). Jacobi *et al.* (*44*) and Rioux *et al.* (*43*) also demonstrated antifungal activities of D4E1 and D2A21 on pathogens of tree species (*Picea, Pinus* and *Populus* species) including *Cronartium ribicola*, *Gremmeniella abietina*, *Melampsora medusae*, *Nectria galligena*, *Ophiostoma ulmi*, and *Septoria musiva*. These two synthetic peptides did not affect pollen germination. Using a transgenic tobacco model system we also reported that the D4E1 gene greatly enhanced disease resistance *in planta* to foliar fungal pathogens *Colletotrichum destructivum (41) and Alternaria spp. (*Rajasekaran, unpublished) and the bacterial pathogen *Pseudomonas syringae* pv. *tabaci* (*65*). Treatment of pre-germinated *A. flavus* spores with tobacco leaf extracts from plants, transformed with the D4E1 gene, resulted in significant reduction in spore viability (colony forming units) relative to extracts from non-transformed (control) plants. Similarly, in recent tests with cottonseed expressing the D4E1 gene, we demonstrated resistance to penetration of seed coats by a toxigenic *A. flavus* strain that expressed a GFP reporter gene (*64*, *66*). Growth of the fungus and the resultant toxin production were greatly reduced in transgenic plants expressing D4E1. In addition to inhibiting the germination of fungal spores, D4E1 caused severe abnormal lytic effects on mycelial wall, cytoplasm, and nuclei on susceptible species (Figure 3). The expression of D4E1 gene in the progeny of transgenic cotton plants was sufficient enough to inhibit the growth *in vitro* of *Fusarium verticillioides* and *Verticillium dahliae* or *in planta* of *Thielaviopsis basicola* (*64*) and provide a good germination stand in a field infected with

Fusarium oxysporum f.sp. *vasinfectum* (*67*) and other seedling pathogens (Odom *et al.* personal communication). Mentag *et al.* (*46*) demonstrated bacterial disease resistance of transgenic hybrid poplar (*Populus tremula* L. x *Populus alba* L.) expressing the synthetic antimicrobial peptide D4E1. The transgenic poplar lines were tested for resistance to bacterial diseases caused by *Agrobacterium tumefaciens*, *Xanthomonas populi* pv. *populi* and *Hypoxylon mammatum* (Wahl.). One transgenic poplar line, Tr23, bearing the highest transcript accumulation for the D4E1 gene, showed a significant reduction in symptoms caused by *A. tumefaciens* and *X. populi*. However, none of the transgenic poplar lines showed a significant difference in disease response to the fungal pathogen *H. mammatum*.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

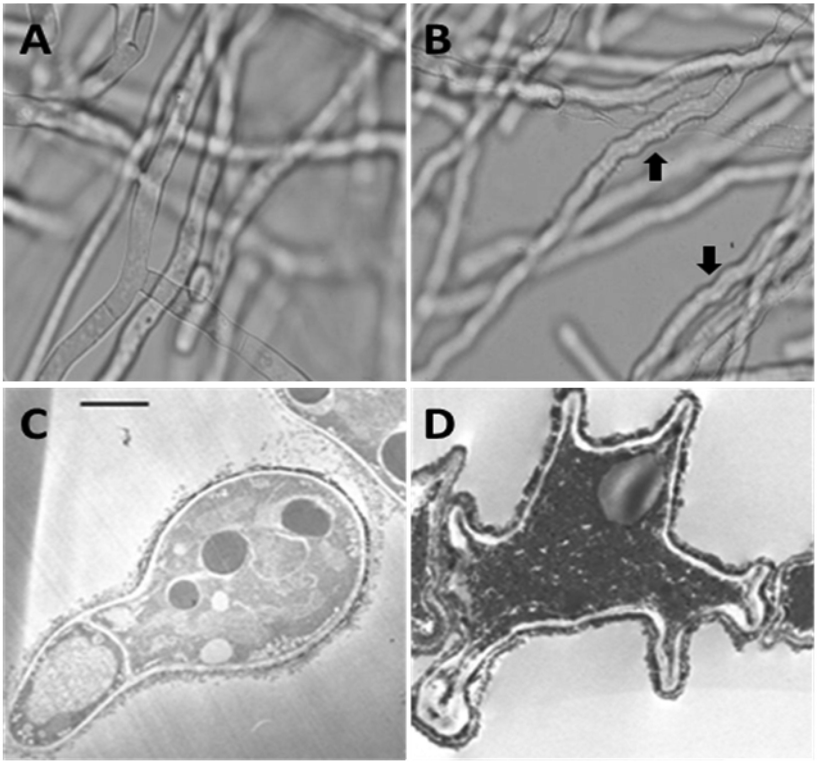


Figure 3. Lytic effect of antimicrobial peptide D4E1 on Rhizoctonia solani and Aspergillus flavus. R. solani hyphae after 48 h in the presence of 0 µM (A) and 20 µM D4E1 (B). Arrows indicate morphological changes in hyphal growth habit in the presence of 20 µM D4E1. Pregerminated spores of A. flavus in the absence of D4E1 (C), and upon treatment with 25 µM D4E1 for 1 h showing lysis (D). Adapted from references (2, 42).

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Transformation of peanut with another synthetic antifungal peptide, D5C1, has been reported (*68*). Although the pure D5C1 showed strong activity *in vitro* against *A. flavus*, the transgenic peanut callus showed poor recovery of plants because of possible phytotoxicity of the peptide. Puterka *et al.* (*69*) observed the biology and behavior of pear psylla (*Cacopsylla pyricola)* feeding on a transgenic clone of *Pyrus communis* expressing a synthetic antimicrobial gene D5C1. The purpose of the original transformation was to enhance pear resistance to the bacterial disease fireblight caused by *Erwinia amylovora* (Burr.). During the course of the study, they observed that the insect pest's biology and behavior were initially enhanced on a transgenic pear clone. However, chronic exposure of psylla populations to transformed pear plants that express the *npt*II marker and lytic peptide genes had detrimental effects on the pear psylla insect pest.

Transgenic expression of a synthetic substitution analog of magainin, MSI-99 imparted disease resistance in both tobacco (*Nicotiana tabacum* L.) and banana [*Musa* spp. cv. Rasthali (AAB)]. Transgenic tobacco showed enhanced resistance to *Sclerotinia sclerotiorum*, *Alternaria alternata* and *Botrytis cinerea* whereas transgenic banana plants showed resistance to *Fusarium oxysporum* f.sp. *cubense* and *Mycosphaerella musicola* (*70*). Alan *et al.* (*71*) transformed tomato with MSI-99 and they observed that transgenic tomato plants were more resistant to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (bacterial speck pathogen) than against the fungal pathogens - *Alternaria solani* (early blight) and the oomycete pathogen *Phytophthora infestans* (late blight) possibly due to proteolytic degradation and lower expression of the peptide. MSI-99 was used to impart enhanced resistance against the fungal pathogen, *Aspergillus niger* in two transgenic potato cultivars according to Ganapathi *et al.* (*47*).

A synthetic derivative of dermaseptin B1, MsrA2 (N-Met-dermaseptin B1), elicited strong antimicrobial activity against various phytopathogenic fungi and bacteria *in vitro* (*72*, *73*). To assess its potential for plant protection, MsrA2 was expressed at low levels (1-5 microgram/g of fresh tissue) in the transgenic potato (*Solanum tuberosum* L.) cv. Desiree. Stringent challenges of these transgenic potato plants with a variety of highly virulent fungal phytopathogens - *Alternaria, Cercospora, Fusarium, Phytophthora, Pythium, Rhizoctonia* and *Verticillium* species-and with the bacterial pathogen *Erwinia carotovora* (syn. *Pectobacterium carotovorum)* demonstrated that the plants had an unusually broad-spectrum and powerful resistance to infection. These authors reported that MsrA2 protected both plants and tubers from diseases such as late blight, dry rot, and pink rot and markedly extended the storage life of tubers.

Non-Nuclear Expression of Synthetic Peptides in Transgenic Plants

Nuclear transformation methods have been successfully used to genetically engineer a wide variety of crop plants used for food and feed including corn, soybeans and cotton. The most common methods for nuclear transformation in plants are *Agrobacterium*-mediated, biolistic and electroporation-mediated introduction of the foreign DNA. Regardless of the methodology utilized, nuclear

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

transformation has a variety of disadvantages. First, the integration site of the gene of interest is unknown and can lead to highly variable levels of transgene expression (*74*). Second, nuclear transformants are potentially affected by gene silencing events, making it difficult to achieve efficacious levels of transgene products (*75*). Additional factors that need to be considered for optimal expression of transgenes include considerations of promoter strength and expression profile, transcriptional activation signals, transcript stabilization sequences, translational regulation and protein modifications (*76*). Third, it is often preferred to introduce multiple genes for a desirable trait that need to be coordinately expressed in the same plant (stacked genes). This is difficult if each gene uses a different promoter (*77*). Fourth, nuclear transformed plants can express the transgene in pollen which can escape into the general plant population and cause undesirable distribution of the transgene (*78*, *79*).

a. Plastid and Mitochondrial Transformation and Expression of Transgenes

Localization and expression of transgenes in plastids has the potential to circumvent many of the disadvantages associated with nuclear transformation described above (*80*). Plants have multiple types of plastids including chloroplasts, leucoplasts and chromoplasts. To date, the primary target for plastid transformation and expression of transgenes has been the chloroplast; however, all plastids are derived from a common progenitor (proplastid), and strategies for chloroplast transformation and expression should be applicable to all plastid types.

The large number of copies of a transgene that can be localized to a designated location within plastids, the capacity to express multiple genes from a single promoter coupled with the lack of gene silencing makes plastid transformation and expression an attractive system for genetically engineering plants. Within a single tobacco chloroplast, there are approximately 100 copies of the plastid genome, and between 10 and 100 plastids per cell resulting in the potential for harboring between 1000 and 10,000 copies of a transgene per cell (*81*). Because strategies for plastid transformation rely upon homologous recombination between the genes of interest and the plastid genome, the DNA to be transferred is engineered with sequences that are homologous to the plastid genome. Thus, the site of integration is known, and variability due to different sites of integration is eliminated. Another major advantage of plastid transformation results from the prokaryotic-like nature of plastids. Genes in the plastid genome are often expressed in operon-like cassettes. Thus suites of genes can be introduced into a plastid and the expression of all the newly introduced “stacked” genes will be coordinately regulated by a single promoter (*77*). This approach has been successfully used to express the polyhydroxybutyric acid operon from *Ralstonia eutropha* in tobacco chloroplasts (*82*). Additional advantages of plastid based transformation systems are: enhanced transgene containment because plastids are maternally inherited (*83*), and newly synthesized proteins and small peptides are protected from cytoplasmic proteases due to sequestration in plastids. The inherent stability of the protein or peptide can also affect the levels of accumulated protein. Ortigosa *et al.* (*84*) found that the amount of a small 21 amino acid peptide expressed in transgenic tobacco chloroplasts was enhanced (from non-detectable levels to 2% of the total soluble protein) when it was expressed as a fusion with the p53 oligomerization domain.

Chloroplast transformation is routine in tobacco, and transplastomic tobacco plants have been developed that express a variety of proteins for a wide variety of uses (*80*, *85–87*). For example, toxin subunits for vaccines such as the cholera toxin have been expressed at high levels in tobacco chloroplasts (*88*). Plastid transformation has also been used to develop crops with enhanced agronomic characteristics. Transplastomic plants that express 5-enolpyruvylshikimate-3-phosphate synthase were more resistant to glyphosate than nuclear transformants that expressed the same gene (*89*). Plastid expression of an analog of magainin-2, MSI-99, a 22 amino acid lytic peptide resulted in tobacco plants with increased resistance to *Aspergillus flavus*, *Fusarium verticillioides* (Syn. *F. moniliforme)*, *Verticillium dahliae*, and *Pseudomonas syringae* pv. *tabaci in vivo* and *in vitro* (*33*). Recently antimicrobial peptides retrocyclin-101 and protegrin-1 were expressed in tobacco chloroplasts to confer resistance to the bacterial pathogen, *Erwinia carotovora* and/or tobacco mosaic virus (*90*). Many other studies have been reviewed in the literature (*87*, *91–94*), and these studies have demonstrated "proof of concept" for the use of plastid expression to generate high levels of functional transgene expression in laboratory, greenhouse and field trials. However, at present, there are no commercially grown transplastomic crops (*87*) largely due to lack of availability of suitable selectable markers.

Mitochondria are also targets for transgene integration and expression (*95*). In humans, mitochondrial transformation has been selected as one approach for gene therapy of mitochondrial genetic defects (*96*). In plants, mitochondria have been transformed by electroporation (*97*) or direct DNA uptake (*98*) and used to study RNA processing. *Chlamydomonas* mitochondria have been successfully transformed to introduce mitochondrial sequences that rescue mutations (*99*) but efforts to stably express transgenes in plant mitochondria are still in their infancy.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

b. Targeting Products to Organelles

One of the problems associated with expression of small peptides or proteins in transgenic plants is degradation due to cellular proteases. As opposed to direct expression in the plastids, transgenes can be integrated into the nuclear genome, transcribed in the nucleus, the RNA exported to the cytoplasm and translated in the cytoplasm. If the transgenes are designed so that the proteins and peptides they encode contain signals that target their location to specific cellular structures, the proteins will be protected from cytoplasmic proteases. For example, nuclear encoded transgenes have been designed so that the protein products contain signal sequences that target them to plastids (*100*), mitochondria (*101*), peroxisomes (*102*), seed oil bodies (*103*), protein bodies (*104*, *105*) or endoplasmic reticulum (*106*). Proteins can also be specifically targeted to two different cellular locations, such as chloroplasts and peroxisomes by incorporating the appropriate targeting signals (*107*).

In some cases, transgene product accumulation is limited due to cytotoxic effects. These effects can be mitigated by sequestering the potentially toxic molecules in membrane bound organelles or vesicles. Sequestration of protein and peptide transgene products also separates any potentially toxic peptides and proteins from cellular processes.

c. Transgenic Expression: Promoter Choice, Regulatory Elements

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Promoter choice and additional regulatory elements dramatically affect the levels, specificity and stability of the expressed gene products. Various types of promoters such as native, constitutive, tissue-specific, inducible, synthetic or minimal promoters for engineering increased disease resistance in plants are discussed in detail by Gurr and Rushton (*108*). According to these authors, earlier attempts to boost disease resistance in transgenic plants used constitutive overexpression of defense components but frequently resulted in poor quality plants. Commonly used promoters for constitutive nuclear expression include the Cauliflower Mosaic Virus 35S (CaMV 35S), octopine and nopaline synthase, actin and ubiquitin promoters. These promoters are expressed in most tissues at relatively high levels. Synthetic promoters have been assembled that are composed of multiple repetitions of some of these components along with ancillary activating sequences. For example, Ni *et al.* (*109*) developed a “superpromoter” that contained three repeats of the octopine synthase activating sequence (*ocs* UAS) linked to a single copy of the mannopine synthase 2’ activator/promoter. Expression levels achieved with this promoter linked to a GUS reporter gene were up to 156 fold higher than those observed with the single CaMV 35S promoter and 26 fold higher than a double CaMV 35S construct in transgenic tobacco leaves. The “superpromoter” was further modified to make it more amenable for cloning (*110*). In addition to the promoter sequences *per se*, other regulatory sequences such as enhancers and even introns can affect the level and tissue specificity of expression. Histochemical detection of GUS gene expression driven by the superpromoter showed that addition of the *ocs* UAS repeats resulted in higher levels of expression in a wide variety of cell types including leaf, xylem, phloem, root tip and root hairs (*109*). Intron Mediated Enhancement (IME) is sequence and position dependant. Levels of enhancement can exceed 100 fold in monocots (*111*), but are much less pronounced in dicots (*112*, *113*). In studies with the rice ubi3 promoter and its 5’ exon fragment, IME affects transcription, post-transcriptional modification and translation with the greatest effect occurring post-transcriptionally (*114*).

An alternative to the development of promoters for high levels of generalized expression in all tissues is to utilize promoters that will drive expression of the gene of interest in the tissues in which it will have the greatest effect. For example, if resistance to fungal infection is desired, it is logical to express antifungal genes in the tissues subject to fungal attack. This approach involves the selection of promoter elements that are regulated either temporally or spatially. Promoters such as a steroid inducible promoter (*115*) that can be selectively regulated are also viable options. The use of homologous promoters and tightly controlled expression patterns may result in transgenic crops that are more acceptable to the general public.

Plastid expression usually involves the use of promoters and terminators found within the plastid genome. The most commonly used promoter fragment for plastid transformation studies is the rRNA operon (rrn16) promoter because it is a strong promoter (*87*). This promoter is expressed at very high levels in multiple tissues under different developmental stages. However, because the native gene product, rRNA is not translated, the promoter fragment used for plastid transformation/expression studies is fused to a translation regulatory signal (*116*). Other promoters may be more desirable depending on the application. For example, if light-regulated expression of the gene product is desirable, then a promoter associated with a light-regulated gene or genes, such a those associated with the different photosystem components would be appropriate (e.g. *psb*A).

Both plastids and mitochondria share ancestral origins with the Eubacteria. Some plant plastid promoter and all mitochondrial promoters are transcribed by a phage type RNA polymerase. Mitochondrial promoters can be recognized and transcribed in plastids (*117*). An advantage associated with using mitochondrial promoters as opposed to endogenous plastid promoters is that deletions and rearrangements from recombination between homologous plastid sequences and the expression cassette will not occur.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Conclusions

The use of synthetic peptides in transgenic plants to control attack by microbial pathogens is well established based on publications cited in this chapter. The attractiveness of using synthetic peptides to control plant disease is based on the following considerations: - 1) Modern technology in automated peptide synthesis and combinatorial chemistry makes the task of designing novel, environmentally benign, yet target pathogen(s)-specific potent peptides more attainable than before; 2) Potent antifungal peptides are available that are effective against a wide range of plant pathogens including toxin producing fungal (*Aspergillus*, *Fusarium* and others) and bacterial species. Use of such peptides is extremely valuable to combat microbial pathogens that compromise food and feed safety; they can provide broad-spectrum control due to their lytic activity; 3) Availability of transgenic technology in several crops via both nuclear and/or organelle transformation (*2*, *3*, *118*) allows rapid development of disease-resistant, commercially-useful germplasm or varieties of food and feed crops; 4) Introduction of AMPs relies on the combination of well-established technologies to enhance host-plant resistance of susceptible crop species in a much shorter time frame than conventional breeding; 5) Beneficial microbial communities are not expected to be significantly affected by transgenic lines expressing synthetic peptides (*119*, *120*) because they have a short half-life and are largely contained in the plant tissue.

References

1. Marcos, J. F.; Muñoz, A.; Pérez-Payá, E.; Misra, S.; López-García, B. *Annu. Rev. Phytopathol.* **2008**, *46*, 273–301.
2. Rajasekaran K.; Jaynes J. M.; Cary J. W. In *Mycotoxin Prevention and Control in Agriculture*; Appell, M., Kendra, D. F., Trucksess, M. W., Eds.; American Chemical Society: Washington, DC, 2009; Vol. 1031, pp 119−142.
3. Montesinos, E. *FEMS Microbiol. Lett.* **2007**, 1–11.
4. Cary, J. W.; Rajasekaran, K.; Brown, R. L.; Luo, M.; Chen, Z.-Y.; Bhatnagar, D. *Toxins* **2011**, *3*, 678–696.
5. Riley R. T. In *Mycotoxins in Agriculture and Food Safety*; Sinha, K. K., Bhatnagar, D. Eds.; Marcel Dekker: New York, 1998; pp 227−253.
6. Cohen, S. M.; Ellwein, L. B. *Science* **1990**, *249*, 1007–1011.
7. IARC: Int Agency Res Cancer *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; **1993**, *56*, 245−395.
8. Rustom, I. Y. S. *Food Chem.* **1997**, *59*, 57–67.
9. CDC. *MMWR Morb Mortal Wkly Rep* **2004**, *53*(34), 790–3. Available from: http://www.cdc.gov/nceh/hsb/chemicals/pdfs/mmwr5334p790.pdf . Accessed 2011 Sep 1.
10. Murphy, P. A.; Hendrich, S; Landgren, C; Bryant, C. M. *J. Food Sci.* **2006**, *71*, R51–65.
11. Hsieh D. P. H. In *Mycotoxins and Phycotoxins*; Natori, S., Hashimoto, K., Ueno Y., Eds.; Elsevier, Biomedical Press: Amsterdam, 1989; pp 69−80.
12. Jackson, E. W.; Wolf, H.; Sinnhuber, R. O. *Cancer Res.* **1968**, *28*, 987–991.
13. Bonomi, A.; Quarantelli, A.; Mazzali, I.; Cabassi, E.; Corradi, A.; Lecce, R.; Ubaldi, A.; Fusari, A.; Chizzolini, A. *Riv. Sci. Aliment.* **1994**, *23*, 251–277.
14. Chu, F. S.; Bhatnagar, D. In *Fungal Biotechnology in Agricultural, Food, and Environmental Applications*. Mycology Series, 21. Arora, D. K., Bhatnagar, D., Eds.; Marcel Dekker, Inc.: New York, 2004; pp 325−342.
15. Gong, Y. Y.; Cardwell, K.; Hounsa, A.; Egal, S.; Turner, P. C.; Hall, A. J.; Wild, C. P. *Brit. Med. J.* **2002**, *325*, 20–21.
16. Nichols, T. E., Jr. *South Coop Ser Bull. 279*. Diener, U. L., Asquith, R. L., Dichens, J. W., Eds.; Alabama Agr. Exp. Station: Auburn, 1983; pp 67−71.
17. Smith J. E.; Moss M. O. *Mycotoxins: Formation Analyses and Significance*; John Wiley and Sons: Chichesster, NY, 1985.
18. Cleveland, T. E.; Bhatnagar, D. In *Molecular approaches to improving food quality and safety*; Bhatnagar, D., Cleveland, T. E., Eds.; Van Nostrand Reinhold: New York, 1992; pp 205−228.
19. CAST: Council of Agricultural Science and Technology, Task Force Report No. 139. *Mycotoxins: Risks in Plant, Animal, and Human Systems*; 2003.
20. Vardon P. J.; McLaughlin C.; Nardinelli C. In*: Potential Economic Costs of Mycotoxins in the United States*; CAST Task Force Report No. 139; 2003, pp 136−142.
21. Robens, J.; Cardwell, K. *J. Toxicol. (Toxin Rev.)* **2003**, *22*, 139–152.
22. Charmley, L. L.; Trenholm, H. L.; Preluskey, D. B.; Rosenberg, A. *Nat. Toxins* **1995**, *3*, 199–203.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

23. Chen, Z.-Y.; Rajasekaran, K.; Brown, R. L.; Bhatnagar, D.; Cleveland, T. E. In*: Plant Genetic Engineering*; Jaiwal, P. K., Singh, R. P., Eds.; Studium Press LLC: Houston, TX, 2006; Vol. 8, pp 73−110.
24. Bhatnagar, D.; Rajasekaran, K.; Brown, R. L.; Cary, J. W.; Yu, J.; Cleveland, T. E. In *Microbial Food Contamination*; Wilson, C. L., Ed.; CRC Press: Boca Raton, FL, 2007; pp 395−426.
25. Bhatnagar, D.; Rajasekaran, K.; Cary, J. W.; Brown, R. L.; Yu, J.; Cleveland, T. E. In *Mycotoxins: Detection Methods, Management, Public Health, and Agricultural Trade*; Leslie, J. F., Bandyopadhyay, R., Visconti, A., Eds.; CABI Publishing: Wallingford, Oxfordshire, U.K., 2008; pp 257−276.
26. Bhatnagar, D.; Rajasekaran, K.; Payne, G. A.; Brown, R. L.; Yu, J.; Cleveland, T. E. *World Mycotoxin J.* **2008**, *1*, 3–12.
27. Zasloff, M. *Nature* **2002**, *415*, 389–395.
28. Hancock, R. E. W.; Sahl, H.-G. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.
29. Jaynes, J. M.; Nagpala, P.; Destefano-Beltran, L.; Huang, J. H.; Kim, J. H.; Denny, T; Cetiner, S. *Plant Sci.* **1993**, *89*, 43–53.
30. Norelli, J. L.; Aldwinckle, H. S.; Destefano-Beltran, L; Jaynes, J. M. *J. Cell Biochem.* **1994**, *18A*, 89.
31. Coca, M.; Peñas, G.; Gómez, J.; Campo, S.; Bortolotti, C.; Messeguer, J.; San Segundo, B. *Planta* **2006**, *223*, 392–406.
32. Alan, A. R.; Earle, E. D. *Mol. Plant-Microbe Interact.* **2002**, *15*, 701–708.
33. DeGray, G.; Rajasekaran, K.; Smith, F.; Sanford, J; Daniell, H. *Plant Physiol.* **2001**, *127*, 852–862.
34. Lee, J.-Y.; Boman, A.; Sun, C.; Andesson, M.; Jornvall, H.; Mutt, V.; Boman, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9159–9162.
35. Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449–5453.
36. Haberman, E. *Science* **1972**, *177*, 314–322.
37. Jaynes, J. M.; Xanthopoulos, D.; Destefano-Beltran, L.; Dodds, J. H. *BioEssays* **1987**, *6*, 263–270.
38. Jaynes, J. M. *Drug News Perspect.* **1990**, *3*, 69–78.
39. Thayer, A. *C&E News* **2011**May30.
40. Destefano-Beltran, L.; Nagpala, P. G.; Cetiner, S. M.; Denny, T.; Jaynes J. M. In *Biotechnology in Plant Disease Control*; Chet, I., Ed.; Wiley: New York, 1993; pp 175−189.
41. Cary, J. W.; Rajasekaran, K.; Jaynes, J. M.; Cleveland, T. E. *Plant Sci.* **2000**, *153*, 171–180.
42. Rajasekaran, K.; Stromberg, K.; Cary, J. W.; Cleveland, T. E. *J. Agric. Food Chem.* **2001**, *49*, 2799–2803.
43. Rioux, D.; Jacobi, V.; Simard, M.; Hamelin, R. C. *Can. J. Bot.* **2000**, *78*, 462–471.
44. Jacobi, V.; Plourde, P. J.; Charest, P. J.; Hamelin, R. C. *Can. J. Bot.* **2000**, *78*, 455–461.
45. Weissinger, A.; Wu, M.; Wang, X.; Isleib, T.; Shew, B.; Payne, G.; Rajasekaran, K.; Cary, J.; Cleveland, T. E. *Proc. 2007 Annual Multicrop Aflatoxin/ Fumonisin Elimination & Fungal Genomics Workshop* **2007**, 106–107.

46. Mentag, R.; Luckevich, M.; Morency, M. J.; Seguin, A. *Tree Physiol.* **2003**, *23*, 405–411.
47. Ganapathi, T. R.; Ghosh, S. B.; Laxmi, N. H. S.; Bapat, V. A. *Ind. J. Biotechnol.* **2007**, *6*, 63–67.
48. Hale, J. D.; Hancock, R. E. *Expert Rev. Anti. Infect. Ther.* **2007**, *5*, 951–959.
49. Yeaman, M. R.; Yount, N. Y. *Pharmacol. Rev.* **2003**, *55*, 27–55.
50. De Lucca, A. J. *Expert Opin. Invest. Drugs* **2000**, *9*, 273–299.
51. Tossi, A.; Sandri, L.; Giangaspero, A. *Biopolymers* **2000**, *55*, 4–30.
52. Lohner, K *Gen. Physiol. Biophys.* **2009**, *28*, 105–116.
53. Chen, Y.; Vasil, A. I.; Rehaume, L.; Mant, C. T.; Burns, J. L.; Vasil, M. L.; Hancock, R. E.; Hodges, R. S. *Chem. Biol. Drug Des.* **2006**, *67*, 162–173.
54. Shai, Y. *Biopolymers (Peptide Sci)* **2002**, *66*, 236–248.
55. De Lucca, A. J.; Bland, J. M.; Grimm, C; Jacks, T. J.; Cary, J. W.; Jaynes, J. M.; Cleveland, T. E.; Walsh, T. J. *Can. J. Microbiol.* **1998**, *44*, 514–520.
56. Carvalho Ade, O.; Gomes, V. M. *Peptides* **2009**, *30*, 1007–1020.
57. Hwang, B.; Hwang, J. S.; Lee, J.; Lee, D. G. *Biochem. Biophys. Res. Commun.* **2011**, *405*, 267–271.
58. Brogden, K. A. *Nat. Rev. Microbiol.* **2005**, *3*, 238–250.
59. Marcos, J. F.; Gandia, M. *Expert Opin. Drug Discovery* **2009**, *4*, 659–671.
60. Park, C.; Lee, D. G. *Biochem. Biophys. Res. Commun.* **2010**, *394*, 170–172.
61. Aerts, A. M.; Carmona-Gutierrez, D.; Lefevre, S.; Govaert, G.; Francois, I. E.; Madeo, F.; Santos, R.; Cammue, B. P.; Thevissen, K. *FEBS Lett.* **2009**, *583*, 2513–2516.
62. Lobo, D. S.; Pereira, I. B.; Fragel-Madeira, L.; Medeiros, L. N.; Cabral, L. M.; Faria, J; Bellio, M.; Campos, R. C.; Linden, R.; Kurtenbach, E. *Biochemistry* **2007**, *46*, 987–996.
63. van der Weerden, N. L.; Lay, F. T.; Anderson, M. A. *J. Biol. Chem.* **2008**, *283*, 14445–14452.
64. Rajasekaran, K.; Cary, J. W.; Jaynes, J. M.; Cleveland, T. E. *Plant Biotechnol. J.* **2005**, *3*, 545–554.
65. Rajasekaran, K.; Cary, J. W.; Jacks, T. J.; Cleveland, T. E. In *Crop Biotechnology*; Rajasekaran, K., Jacks, T. J., Finley, J. W., Eds.; ACS Symposium Series No. 829; American Chemical Society: Washington, DC, 2002; pp 97−117.
66. Rajasekaran, K.; Cary, J. W.; Cotty, P.; Cleveland, T. E. *Mycopathol* **2008**, *165*, 89–97.
67. Rajasekaran, K.; Ulloa, M.; Hutmacher, R.; Cary, J. W.; Cleveland, T. E. *Proc 2006 Beltwide Cotton Conferences*; National Cotton Council: Memphis, TN, 2006; pp 895−903.
68. Weissinger, A.; Liu, Y.-S.; Scanlon, S.; Murray, J.; Cleveland, T. E.; Jaynes, J; Mirkov, E.; Moonan, F. *Proc USDA-ARS Aflatoxin Elimination Workshop* **1999**, 66–68.
69. Puterka, G. J.; Bocchetti, C.; Dang, P.; Bell, R. L.; Scorza, R. *J. Econ. Entomol.* **2002**, *95*, 797–802.
70. Chakrabarti, A.; Ganapathi, T. R.; Mukherjee, P. K.; Bapat, V. A. *Planta* **2003**, *216*, 587–596.
71. Alan, A. R.; Blowers, A.; Earle, E. D. *Plant Cell Rep.* **2004**, *22*, 388–396.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

72. Osusky, M.; Zhou, G.; Osuska, L.; Hancock, R. E.; Kay, W. W.; Misra, S. *Nat. Biotechnol.* **2000**, *18*, 1162–1166.
73. Osusky, M.; Osuska, L; Kay, W.; Misra, S. *Theor. Appl. Genet.* **2005**, *111*, 711–722.
74. Peach, C.; Velten, J. *Plant Mol. Biol* **.1991**, *17*, 49–60.
75. Bruening, G. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13349–13351.
76. Hudson, L. C.; Bost, K. L.; Piller, K. J. In *Soybean - Molecular Aspects of Breeding*; Sudaric, A., Ed.; InTech: Croatia, 2011.
77. Halpin, C. *Plant Biotech. J.* **2005**, *3*, 141–155.
78. Daniell, H. *Nat. Biotechnol.* **2002**, *20*, 581–586.
79. Hudson, L. C.; Halfhill, M. D.; Stewart, C. N., Jr. *Methods in Molecular Biology, Transgenic Plants. Methods and Protocols*; Springer: New York, 2005; Vol. 286, pp 365−376.
80. Daniell, H.; Kumar, S.; Dufourmantel, N. *Trends Biotechnol.* **2005**, *23*, 238–245.
81. Shaver, J. M.; Bendich, A. J.; Oldenburg, D. J. *Planta* **2006**, *224*, 72–82.
82. Lossl, A.; Bohmert, K.; Harloff, H.; Eibl, C.; Muhlbauer, S.; Koop, H. U. *Plant Cell Physiol.* **2005**, *46*, 1462–1471.
83. Ruf, S.; Karcher, D.; Bock, R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 6998–7002.
84. Ortigosa, S. M.; Fernandez-San Millan, A.; Veramendi, J. *Trans. Res.* **2010**, *19*, 703–709.
85. Mason, H. S.; Warzecha, H.; Mor, T; Arntzen, CJ *Trends Mol. Med.* **2002**, *8*, 324–329.
86. Fischer, R.; Stoger, E.; Schillberg, S.; Christou, P.; Twyman, R. M. *Curr. Opin. Plant Biol.* **2004**, *7*, 152–158.
87. Maliga, P.; Bock, R. *Plant Physiol.* **2011**, *155*, 1501–1510.
88. Daniell, H.; Lee, S. B.; Panchal, T.; Wiebe, P. O. *J. Mol. Biol.* **2001**, *311*, 1001–1009.
89. Ye, G.-N.; Hajdukiewicz, P. T. J.; Broyles, D.; Rodriguez, D.; Xu, C. W.; Nehra, N.; Staub, J. M. *Plant J.* **2001**, *25*, 261–270.
90. Lee, S.-B.; Li, B.; Jin, S.; Daniell, H. *Plant Biotechnol. J.* **2011**, *9*, 100–115.
91. Wani, S. H.; Haider, N.; Kumar, H.; Singh, N. B. *Curr. Gen.* **2010**, *11*, 500–512.
92. Repkova, J. *Czech. J. Gen. Plant Breed.* **2010**, *46*, 103–113.
93. Altpeter, F.; Baisakh, N.; Beachy, R.; Bock, R.; Capell, T.; Christou, P.; Daniell, H.; Datta, K.; Datta, S.; Dix, P. J.; Fauquet, C.; Huang, N.; Kohli, A.; Mooibroek, H.; Nicholson, L.; Nguyen, T. T.; Nugent, G.; Raemakers, K.; Romano, A.; Somers, D. A.; Stoger, E.; Taylor, N.; Visser, R. *Mol. Breed.* **2005**, *15*, 305–327.
94. Clarke, J.; Daniell, H. *Plant Mol. Biol.* **2011**, *76*, 211–220.
95. Ijaz, S. *African J. Biotechnol.* **2010**, *9*, 9196–9199.
96. Minhyung, L.; Joon, S. C.; Min, J. C.; Youngmi, K. P.; Byoung, D. R.; Kyung, S. K. *J. Drug Target* **2007**, *15*, 115–122.
97. Farre, J. C.; Choury, D.; Araya, A. *Methods Enzymol.* **2007**, *424*, 483–500.
98. Placido, .; Gagliardi, D.; Gallerani, R.; Grienenberger, J.-M.; Marechal-Drouard, L. *J. Biol. Chem.* **2005**, *280*, 33573–33579.

Downloaded by PENNSYLVANIA STATE UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch014

99. Remacle, C.; Cardol, P.; Coosemans, N.; Gaisne, M; Bonnefoy, N. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 4771–4776.
100. Corbin, D. R.; Grebenok, R. J.; Ohnmeiss, T. E.; Greenplate, J. T.; Purcell, J. P. *Plant Physiol.* **2001**, *126*, 1116–1128.
101. Yang, J.; Liu, X.; Yang, X.; Zhang, M. *BMC Plant Biol.* **2010**, *10*, 231.
102. Kato, A.; Hayashi, M.; Nishimura, M. *Plant Cell Physiol.* **1999**, *40*, 586–591.
103. Parmenter, D. L.; Boothe, J. G.; Rooijen, G. J. H.; Yeung, E. C.; Moloney, M. M. *Plant Mol. Biol.* **1995**, *29*, 1167–1180.
104. Conley, A.; Joensuu, J.; Menassa, R.; Brandle, J *BMC Biol.* **2009**, *7*, 1–18.
105. Mainieri, D.; Rossi, M.; Archinti, M.; Bellucci, M.; De Marchis, . F; Vavassori, S.; Pompa, A.; Arcioni, S.; Vitale, A. *Plant Physiol.* **2004**, *136*, 3447–3456.
106. Ohara, K.; Kokado, Y.; Yamamoto, H.; Sato, F.; Yazaki, K. *Plant J.* **2004**, *40*, 734–743.
107. Hyunjong, B.; Lee, D.-S.; Hwang, I. *J. Exp. Bot.* **2006**, *57*, 161–169.
108. Gurr, S.; Rushton, P. J. *Trends Biotechnol.* **2005**, *23*, 283–289.
109. Ni, M.; Cui, D.; Einstein, J.; Narasimhulu, S; Vergara, C. E.; Gelvin, S. B. *Plant J.* **1995**, *7*, 661–676.
110. Lee, L.-Y.; Kononov, M. E.; Bassuner, B.; Frame, B. R.; Wang, K.; Gelvin, S. B. *Plant Physiol.* **2007**, *145*, 1294–1300.
111. Clancy, M.; Hannah, L. C. *Plant Physiol.* **2002**, *130*, 918–929.
112. Rose, A. B.; Beliakoff, J. A. *Plant Physiol.* **2000**, *122*, 535–542.
113. Rose, A. B. *RNA* **2002**, *8*, 1444–1453.
114. Samadder, P. S. E.; Lu, J; Li, X.; Qu, R. *Mol. Gen. Genom.* **2008**, *4*, 429–439.
115. Schena, M.; Lloyd, A.; Davis, R. *Genes Dev.* **1993**, *7*, 367–379.
116. Maliga, P. *Curr. Opin. Plant Biol.* **2002**, *5*, 164–172.
117. Bohne, A.-V.; Ruf, S.; Borner, T.; Bock, R. *Nucleic Acids Res.* **2007**, *35*, 7256–7266.
118. Rajasekaran, K.; Bhatnagar, D.; Brown, R. L.; Chen, Z.-Y.; Cary, J. W.; Cleveland, T. E. In *Perspectives of Agricultural Research and Development*; Ramasamy, C., Ramanathan, S., Dhakshinamoorthy, M., Eds.; Tamil Nadu Agricultural University Centennial Issue: Coimbatore, India, 2005; pp 434−467.
119. Stewart, J. McD.; Nader, C. A.; Rajasekaran, K. University of Arkansas Cotton Research 2007. *AAES Research Series 562* **2007**, 163–166.
120. O’Callaghan, M.; Gerard, E. M.; Waipara, N. W.; Young, S. D.; Glare, T. R.; Barrell, P. J.; Conner, A. J. *Plant Soil* **2004**, *266*, 47–56.
121. Jaynes, J.; Bernard, G. In *Small Wonders: Peptides for Disease Control*; Rajasekaran, K.; Cary, J. W.; Jaynes, J. M.; Montesinos, E. ACS Symposium Series 1095; American Chemical Society: Washington, DC, 2012.
122. Mejia, L. C.; Guiltinan, M. J.; Shi, Z.; Landherr, L.; Maximova, S. N. In *Small Wonders: Peptides for Disease Control*; Rajasekaran, K.; Cary, J. W.; Jaynes, J. M.; Montesinos, E. ACS Symposium Series 1095; American Chemical Society: Washington, DC, 2012.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Chapter 15

Antifungal Plant Defensins: Structure-Activity Relationships, Modes of Action, and Biotech Applications

Uma Shankar Sagaram,[1] Jagdeep Kaur,[1] and Dilip Shah[*]

Donald Danforth Plant Science Center, St. Louis, Missouri 63132, U.S.A.
[*]Email: dshah@danforthcenter.org
[1]Authors have made equal contribution.

Small cysteine-rich antimicrobial proteins known as defensins are expressed in all plants. Although structurally similar, plant defensins exhibit substantial variation in their amino acid sequences. Some of these defensins display potent antifungal activity against fungal and oomycete pathogens making them attractive candidates for expression in transgenic crops for enhanced disease resistance. Significant advances have been made recently in elucidating structural motifs that are essential for their antifungal activity. Our current knowledge of the structure-activity relationships of these proteins will facilitate a rational design of more potent antifungal proteins with low toxicity against non-target organisms. Recent studies have also provided new insights into the different modes of antifungal action of plant defensins. While some defensins require binding to specific plasma membrane localized sphingolipids for their antifungal action, others are translocated into the cytoplasm of fungal cells and require fungal cell wall for their antifungal action. The molecular mechanisms of the entry of defensins into fungal cells and their intracellular targets remain to be elucidated. Greater understanding of the structure-activity relationships and modes of antifungal action of plant defensins will facilitate engineering crops for more robust and durable resistance to fungal and oomycete pathogens in future.

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Introduction

Fungal pathogens severely limit agricultural production worldwide. It is estimated that approximately 10% of crop yields are lost to these pathogens (*1*). Many cultivated crops have minimal resistance to these pathogens and require intensive inputs of chemical fungicides for disease management. Improving resistance to these pathogens has been constrained by the lack of resistant germplasm and the difficulty of introgressing limited sources of resistance into the elite germplasm without linkage drag. Novel sources of effective and durable resistance to these pathogens must be developed in order to sustain crop production. Moreover achieving commercially useful level of disease resistance is further impeded by complex interplay between classical disease triangle consisting of host, pathogen and environment. In order to protect themselves from invading pathogens, plants have developed numerous countermeasures which result in the activation of a plethora of defense mechanisms both locally at the infection site and systemically throughout the plant. These plant defense mechanisms include cell wall reinforcements, production of reactive oxygen species (ROS), synthesis of antimicrobial metabolites, pathogenesis-related (PR) proteins and antimicrobial peptides. These defense mechanisms are part of the innate immunity of plants and successful pathogens overcome them to colonize plants and establish disease. Transgenic approaches to augment the plant's innate defense mechanisms are being pursued aggressively to generate disease resistant crops. These approaches among others include expressing antifungal proteins in transgenic crops. Plants express a number of antimicrobial proteins during their normal growth and development and in response to challenge by various pathogens. Among those are the well-characterized small cysteine-rich proteins known as defensins that exhibit potent antifungal activity (*2*, *3*). Defensins are thought to be the early effectors of innate immunity in plants and animals (*2*, *4*–*6*). Based on their remarkable structural similarity, it has been suggested that defensins have likely evolved from a common ancestor (*2*). Defensins differing significantly in their amino acid sequences are produced by plants during their normal lifecycle and in response to a variety of biotic or abiotic stimuli. Many of these defensins have been reported to be potent inhibitors of various fungi and thus have the potential to be used as antifungal agents in transgenic crops. Studies of defensins are of great interest both for mechanistic knowledge of this important class of plant defense proteins as well as for advancing crop improvement. Here, we review our current knowledge of the structure-activity relationships and modes of antifungal action of these proteins. We also describe the progress made in engineering resistance to fungal pathogens in transgenic plants through ectopic expression of defensin genes.

Structure, Expression, and Antifungal Activity of Plant Defensins

Plant defensins are small cysteine-rich proteins of 45-54 amino acids and show structural similarity to insect and mammalian defensins (*2*–*5*). Several plant defensins have been purified and their three-dimensional structures have been determined (*7–13*). They share an identical backbone structure which is comprised of one α-helix and three antiparallel β-strands and is stabilized by the presence of a highly conserved tetradisulfide array. The presence of an additional 5th disulfide bond has also been noted in a floral defensin of *Petunia hybrida* (*11*). As of now, amino acid sequences of over 100 plant defensins have been deduced from their nucleotide sequences. These sequences have revealed a remarkable sequence variation in their primary amino acid sequences outside the highly conserved eight cysteine residues. A large family of defensin genes exists in several plant species. For example, *Arabidopsis thaliana* has a family of 13 different defensin genes (*2*), whereas a model legume *Medicago truncatula* which was earlier reported to have a family of 16 defensin genes (*14*) now has more than 20 genes (Kaur and Shah, unpublished data). Homologs of defensin MsDef1 from *M. sativa* are present only in legumes, whereas homologs of MtDef4 from *M. truncatula* are present in many legume and nonlegume plants (*15*). A vast majority of plant defensins are synthesized as precursor proteins containing the secretory signal peptide sequence and the mature defensin peptide sequence. Although these defensins are presumed to be secreted to the extracellular space, an exception has been recently reported. AhPDF1.1 defensin from *A. halleri* is retained in the intracellular compartments in spite of the presence of the secretory signal peptide sequence, thus subcellular targeting may explain the dual role of defensins on plant cells (*16*). A few floral defensins with an additional carboxy-terminal domain are targeted to the vacuole (*12*). It is likely that localization of defensins in specific subcellular compartments is important to minimize their potentially harmful effects on normal growth and development of plants and to maximize their contribution to plant defense against fungal pathogens.

Although defensins were first reported to be abundant in seeds, expression analysis has revealed that they are expressed in every organ of a plant. For example, at least one defensin gene is expressed in every organ of *A. thaliana*, while two or more genes are expressed in some organs (*2*). Preferential expression of defensin genes in the peripheral cell layers of certain tissues strongly suggests their role in plant defense against pathogen attack. Apart from their constitutive expression during normal growth and development of a plant, defensin genes are also induced in response to a range of biotic and abiotic stimuli. For instance, plant defensin expression is either induced or repressed in response to hormones (*17*, *18*), cold treatment (*19*, *20*), drought (*21*), zinc tolerance (*22*), wounding (*23*), mycorrhizal or rhizobial symbiosis (*14*), and pathogens (*24–28*). In addition to their roles in plant defense against fungal pathogens, plant defensins have also been shown to play other biological roles which include antibacterial activity (*29–32*), zinc tolerance (*22*), proteinase inhibitory activity (*33*), α-amylase inhibitory activity (*34*), ion channel blocking activity (*35–37*) and pollen tube growth arrest, burst and sperm discharge (*37*). Alternative biological functions of plant defensins have also been described in recent reviews (*38*, *39*).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Table I. List of plant defensins effective against a diverse range of plant pathogens (fungi, yeasts, and oomycete) documented in literature

Plant defensin	*Plant origin*	*Plant pathogen*	*Reference*
RsAFP2	*Raphanus sativus*	*Magnaporthe oryzae* *Rhizoctonia solani* *Fusarium culmorum* *F. oxysporum* *Nectria haematococca* *Botrytis cinerea* *Verticillium dahliae* *Mycosphaerella fijiensis*	(*42*)
HsAFP1	*Heuchera sanguinea*	*F. culmorum*	(*43*)
Pdf1.2	*Arabidopsis thaliana*	*Alternaria brassicicola*	(*24*)
Tfgd1	*Trigonella foenum-graecum*	*R. solani* *Phaeoisariopsis personata*	(*94*)
MtDef4	*Medicago truncatula*	*F. graminearum* *F. verticillioides* *F. proliferatum* *F. pseudograminearum*	(*40*) J. Kaur, U. Sagaram and D. Shah, unpublished data
VvAMP1	*Vitis vinifera*	*B. cinerea* *F. solani* *F. oxysporum* *V. dahliae*	(*95*)

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Plant defensin	***Plant origin***	***Plant pathogen***	***Reference***
MsDef1 (previously called alfAFP)	*Medicago sativa*	*V. dahliae* *F. graminearum*	(*40*, *41*)
Dm-AMP1	*Dahlia merckii*	*Neurospora crassa*	(*64*)
Rice bean antifungal peptide	*Delandia unbellata*	*Mycosphaerella arachidicola* *F. oxysporum* *R. solani, B. cinerea* *Colletotrichum gossypii*	(*98*)
BSD1	*Brassica campestris* L. *ssp. pekinensis*	*P. parasitica, N. crassa* *F. oxysproum, A. solani*	(*99*)
PpDfn1	*Prunus persica*	*Penicillium expansum* *B. cinerea*	(*100*)
VaD1	*Vigna radiata*	*F. oxysporum*	(*101*)
LcDef	*Lens culinaris*	*Aspergillus niger*	(*96*)
Ec-AMP-D1 Ec-AMP-D2	*Echinochloa crusgalli*	*Phytophthora infestans*[1]	(*97*)
NaD1	*Nicotiana alata*	*F. oxysporum* f. sp. *vasinfectum*	(*62*)
PDC1	*Corn*	*F. graminearum*	(*105*)
PvD1	*Phaseolus vulgaris*	*Saccharomyces cerevisiae*[a]	(*106*)
lmdef	*Lepidium meyenii*	*P. infestans*[b]	(*107*)

Continued on next page.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Table I. (Continued). List of plant defensins effective against a diverse range of plant pathogens (fungi, yeasts, and oomycete) documented in literature

Plant defensin	***Plant origin***	***Plant pathogen***	***Reference***
Dm-AMP1	*Dahlia merckii*	*Verticillium albo-atrum* *B. cinerea*	(*102*)
Vulgarinin	*Phaseolus vulgaris*	*M. arachidicola* *Physalospora piricola* *F. oxysporum* *B. cinerea*	(*103*)
Antifungal peptide	*Phaseolus limensis*	*F. oxysporum* *M. arachidicola* *Physalospora piricola*	(*104*)
cdef1	*Capsicum annum*	*P. infestans*^{b}	(*108*)
NmDef02	*Nicotiana megalosiphon*	*Peronospora lycopersici* f. sp. *tabacina*^{b} *P. infestans*^{b}	(*80*)
MtDef4	*M. truncatula*	*Hyalopernospora arabidopsidis*^{b}	J. Kaur, A. Robert-Seilaniantz, J. Jones and D. Shah, unpublished data

^{a}yeast; ^{b}oomycete

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

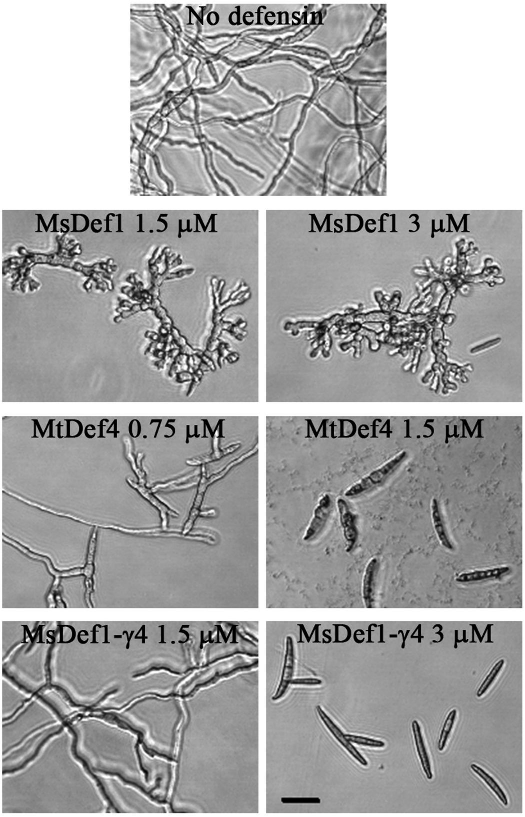


Figure 1. Inhibition of conidial germination and hyphal growth in the presence of MsDef1, MtDef4 and the variant MsDef1-γ4. Conidia of the wild-type PH-1 suspended in synthetic fungal medium were incubated with MsDef1 or MtDef4 or MsDef1-γ4 in the dark. Images were taken after 16 hours of incubation. Bar = 20 μm. Note the hyperbranching of hyphae in the presence of MsDef1 (morphogenic) but not in the presence of MtDef4 (nonmorphogenic). Also note that MsDef1 γ-core substitution variant, MsDef1-γ 4, exhibits antifungal activity similar to that of MtDef4 and also lost the ability to induce hyperbranching. Figure adopted from Sagaram et al., 2011 and modified with permission.

Antifungal Properties of Plant Defensins

Many plant defensins have been shown to inhibit the growth of a broad range of pathogenic fungi, yeast and oomycetes (Table I) *in vitro* at micromolar concentrations, whereas others have no known antifungal activity (*2*, *3*, *38*). Based on the morphological alteration caused by plant defensins to the target fungus, plant defensins can be divided into two subgroups: morphogenic and non-morphogenic (Figure 1). Morphogenic defensins retard fungal hyphal growth

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

with a corresponding increase in hyphal branching while non-morphogenic defensins retard fungal hyphal growth without inducing significant morphological changes to the fungus. Our lab has been investigating the antifungal properties of two plant defensins, MsDef1 (previously called AlfAFP (*40*),) and MtDef4 derived from *M. sativa* and *M. truncatula*, respectively. When tested against the hemibiotrophic fungus *Fusarium graminearum* (strain PH-1), MsDef1 was found to be morphogenic while MtDef4 was non-morphogenic (*41*). Similarly, radish defensins RsAFP1 and RsAFP2 (*42*) and *Heuchera sanguinea* defensin HsAFP1 were previously reported to be morphogenic against *F. culmorum* (*43*). In contrast, Dm-AMP1 from *Dahlia merckii*, Ah-AMP1 from *Aesculus hippocastanum* and Ct-AMP1 from *Clitoria ternatea* turned out to be non-morphogenic when tested against *F. culmorum* (*43*). Because of the difficulty of culturing biotrophic fungi or oomycete pathogens *in vitro*, it has not been possible to determine the antifungal activity of defensins against this important class of pathogens. However, in our lab, we have recently determined that overexpression of MtDef4 confers strong resistance to the obligate oomycete *Hyaloperonospora arabidopsidis* in transgenic *A. thaliana* (Kaur and Shah, unpublished data). Because of the rich diversity of defensin variants that exists in plants, much work remains to be done to fully determine the range of fungi or other pathogens inhibited by these antimicrobial proteins and to unravel their modes of antifungal action.

Structure-Activity Relationships and Modes of Action (MOA) of Plant Defensins

Several plant defensins exhibit broad-spectrum activity against fungi under *in vitro* conditions (*2*, *3*, *38*), whereas several others have no known activity. As explained in the previous section plant defensins with known antifungal activity can be divided into two groups, morphogenic and nonmorphogenic. Interestingly, morphological aberrations caused by morphogenic defensins are detected only in certain sensitive fungi and under particular conditions of fungal growth.

Some progress has been recently made in determining the structural motifs governing the morphogenic or nonmorphogenic effects of a defensin on the fungus. Apart from the cysteine-stabilized αβ (CSαβ) structure, the two most important characteristic features of plant defensins that are evolutionarily conserved include net cationic charge (+2 to +11) and the presence of disulfide bonds. Most plant defensins are stabilized by four intramolecular disulfide bonds. In addition, hydrophobic amino acid residues distributed in such a way that the functional protein attains an amphipathic structure, which might aid in protein solubility in the aqueous as well as lipid environments. Recently, we have reported that antifungal plant defensins, MsDef1 and MtDef4, contain a highly conserved γ-core motif ($GXCX_{3\text{-}9}C$), a structural motif present in the antimicrobial peptides containing disulfide bonds (*44*), composed of β2 and β3 strands and the interposed loop (*15*). The major determinants of the antifungal activity and morphogenicity of these defensins reside in their γ-core motifs, although minor determinants outside the γ-core motifs also contribute to their antifungal activity. We showed that a variant of MsDef1 containing the γ-core motif of MtDef4 has significantly enhanced antifungal activity and becomes a nonmorphogenic defensin (Figure 1). The 16-residue synthetic peptide (GMA1-C) derived from the carboxy-terminus of each defensin and spanning the γ-core motif exhibits substantial antifungal activity (Figure 2). However, interestingly enough, it appears that the 16-mer peptide derived from MsDef1 inhibits fungal growth by a mechanism distinct from that used by the full-length defensin (Figure 2). This hypothesis was further confirmed by using the *F. graminearum* mutants, ΔFg*gcs1* and ΔFg*mgv1*, which are resistant and hypersensitive, respectively, to MsDef1 but not to MtDef4 (*41*, *45*). If both GMA1-C and MsDef1 function in a similar way, then ΔFg*gcs1* must be resistant and Δ*mgv1* must be hypersensitive to GMA1-C. To test this, wild-type *F. graminearum* strain (PH-1) along with mutant strains, ΔFg*gcs1* and Δ*mgv1*, were incubated with different concentrations of MsDef1 and GMA1-C. Interestingly, no significant morphological differences in inhibition of hyphal growth were noticed among PH-1, ΔFg*gcs1* and ΔFg*mgv1* strains in the presence of GMA1-C indicating that GMA1-C and MsDef1 have different modes of action (Figure 2). Further, the 10-residue synthetic peptide containing only the γ-core motif of MtDef4 also retains antifungal activity, but the 9-residue synthetic peptide containing the γ-core motif of MsDef1 does not. This may be due to the fact that the γ-core motif of MtDef4 has a net positive charge of +4, whereas that of MsDef1 has a net positive charge of 0. Further analysis of the γ-core motif of MtDef4 has revealed that RGFRRR peptide derived from it also exhibits potent antifungal activity against *F. graminearum*, but unlike the full-length MtDef4, causes very little permeabilization of the fungal plasma membrane (*15*). Earlier, in radish defensin RsAFP2, two adjacent sites on its 3-D structure were found to be important for its antifungal activity against *F. culmorum* (*46*). One of the regions around the type VI β-turn connecting β-strands 2 and 3 appears to be the highly conserved region similar to the γ-core motifs present in MsDef1 and MtDef4. The structure-activity studies reported thus far suggest that the presence of cationic and hydrophobic amino acids plays an important role in the antifungal activity of plant defensins and the distribution of these amino acids on the peptide determines the potency and specificity. Also, sequence comparison studies revealed that all antifungal plant defensins studied to date have a highly conserved cationic and hydrophobic patch of amino acids in the interposed loop region between β2 and β3 strands strongly suggesting that these conserved patches are most likely important for binding to the fungal membranes and/ or translocation into the cell.

Common to the overall fold in plant defensins is a three-stranded antiparallel β-sheet and an α-helix constrained by four disulfide bridges, Cys1-Cys8, Cys2-Cys5, Cys3-Cys6 and Cys4-Cys7. Currently there is very little information regarding the importance of this tetradisulfide array in providing the stability and/or antifungal activity to the defensin peptide. Although, very little is known regarding the role of disulfide bonds in plant defensins, studies involving mammalian defensins suggest that disulfide bonds have different roles depending upon the environment and target organism. The antimicrobial activity of the human defensin hBD3 remained unaffected in the absence of any disulfide bridge (*47*). Also, this study demonstrated that the disulfide bonding in hBD3 although required for binding and activation of receptors for chemotaxis, was

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

fully dispensable for its antimicrobial function (*47*). Interestingly, a recent study demonstrated that the reduced form of another human β-defensin hBD-1, became a more potent antimicrobial peptide against the opportunistic pathogenic fungus *Candida albicans* and against the anaerobic, Gram-positive *Bifidobacterium* and *Lactobacillus* species, whereas hBD-3 showed decreased activity against *Bifidobacterium* under reduced conditions (*48*). When mouse α-defensin cryptdin-4 (Crp 4) was tested for its antibacterial activity along with several Crp 4 variants lacking various disulfide bonds, all disulfide bond variants had equal or better bactericidal activities. However, all variants were degraded by the MMP-7 protease (*49*) suggesting that the disulfide bonds play an important role in defensin stability rather than its antimicrobial activity. Considering the evolutionary conservation of the four disulfide bonds in plant defensins, it is tempting to think that they might be important for protein stability *in planta*, however, it is important to know which disulfide bonds are essential for the antifungal activity and/or stability of plant defensins. Preliminary data obtained in our lab indicate that the variant of MtDef4 defensin lacking the disulfide bond Cys1-Cys8 is expressed stably in *Pichia pastoris* and retains the near wild-type antifungal activity against *F. graminearum* and *Neurospora crassa*. However, the variants of MtDef4 lacking the Cys2-Cys5, Cys3-Cys6 or Cys4-Cys7 bond are not stably expressed in *P. pastoris* (Sagaram and Shah, unpublished data). It would be interesting to know how the stability and antifungal activity of these variants are affected when expressed *in planta*.

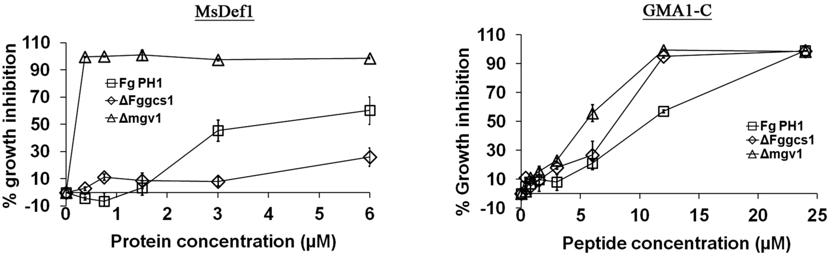


Figure 2. Quantitative assessment of the antifungal activity of MsDef1 and 16-mer synthetic peptide (GMA1-C) derived from MsDef1. Conidia of F. graminearum wild-type strain PH-1 or ΔFggcs1or Δmgv1 were incubated with MsDef1 or GMA1-C for 36 h. The growth was monitored by measuring absorbance at 595 nm. Growth inhibition was measured using the following formula: per cent growth inhibition = [mycelial growth without defensin (control) – mycelial growth with defensin (treatment)/mycelial growth without defensin] × 100. Values are means of three replications. Error bars indicate standard deviations. Note that PH-1 is sensitive to MsDef1 where as ΔFggcs1and Δmgv1 are resistant and hypersensitive, respectively. No such differences were observed in the presence of GMA1-C indicating that it has different modes of action compared to full length MsDef1.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

In addition to elucidating their structure-activity relationships, understanding the modes of antifungal action of plant defensins is critical if we want to use them in transgenic crops for enhanced resistance to fungal pathogens. The three-dimensional structures and antifungal activity of several plant defensins are well characterized. Significant progress has also been made in unraveling the MOA of some of these defensins and these studies have been reviewed elsewhere (*3*, *32*, *50*, *51*). Most plant defensins hitherto identified are toxic to fungal cells but are nontoxic to plant cells indicating that they target unique properties of microbial membranes to provide selectivity in their action. It is a general conviction that positively charged plant defensins bind to negatively charged microbial membranes due to electrostatic attraction. Thus far, three models namely barrel-stave pore, carpet and toroidal pore models have been proposed to explain the attachment and insertion of antimicrobial peptides into the membranes of the target cells (*52*). In the 'barrel-stave model', hydrophobic regions of the peptides align with the lipids of the host membrane while the hydrophilic regions form interior region thus forming a bundle in the membrane with the central lumen (*53*, *54*). In the 'carpet model', peptide molecules cover the surface of the membrane like a carpet (*55*). The initial attachment is due to the electrostatic attraction of peptides to the anionic phospholipid head groups at numerous sites. Peptides are thought to destabilize the bilayer in a detergent-like manner, eventually leading to the formation of micelles (*56*, *57*). In 'toroidal pore model', peptides that are attached to the membrane aggregate and induce the lipid monolayers to bend continuously in such a way that the water core is lined by both the inserted peptides and the lipid heads (*58*). Toroidal pore is thought to be formed by association of the polar faces of the peptides with the polar head groups of the lipids (*59*). It remains largely unknown if plant defensins adopt one of these associations and pore formation mechanisms to permeabilize fungal membranes.

Several studies established that plant defensins bind to the membranes of sensitive bacteria or fungi with high affinity (*60*) and permeabilize them resulting in cell growth arrest (*15*, *61*, *62*). While fungal membrane disruption by plant defensins was shown to be the critical step in antifungal activity (*61*, *63*), the actual mechanism of antifungal action remained unclear. In addition, studies using defensins MsDef1, MtDef4 and their amino acid variants showed that the degree of permeabilization did not correlate with the antifungal potency suggesting that the peptide's ability to permeabilize plasma membrane is not a direct indicator of its antifungal activity (*15*). Based on the work from our lab and others, it is evident that plant defensins have different MOA. Fungal membrane lipids appear to play a critical role in the early steps of the antifungal action of some defensins. For example, DmAMP1 from *Dahlia merckii* binds to an acidic sphingolipid mannosyl diinositolphosphoryl ceramide (*64*), whereas the radish RsAFP2 binds to a neutral sphingolipid glucosylceramide (GlcCer) of the plasma membrane (*65*). MsDef1 also appears to bind to GlcCer of *F. graminearum*, whereas MtDef4 seems to have a different mode of interaction with *F. graminearum* membrane (*45*). A GlcCer synthase knockout mutant of *F. graminearum* (*ΔFggcs1*) displays strong resistance to MsDef1, but remains sensitive to MtDef4 indicating the latter defensin uses GlcCer-independent mechanism of antifungal action. Virtually all defensins that possess antimicrobial activity cause permeability of the host membrane. But, it is not clearly known if permeabilization of fungal membrane is the result of an insertion of a plant defensin into the membrane or the activation of an endogenous sphingolipid-mediated signaling pathway. Molecular mechanisms of fungal growth inhibition following the initial binding of the defensin with sphingolipid remain poorly understood. It is likely that the defensin/sphingolipid interaction results in the activation of signaling pathways which result in fungal growth arrest. The possibility that defensin is internalized by the fungal cell following its interaction with the sphingolipid molecule cannot be ruled out.

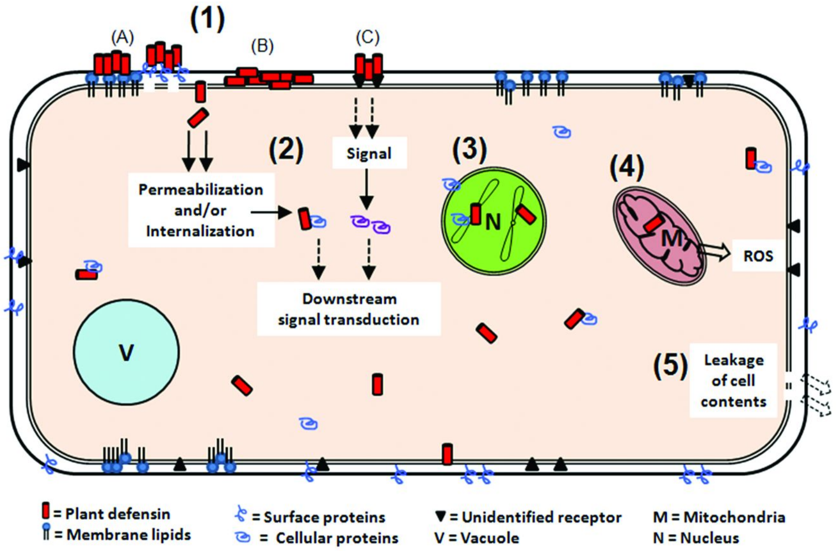

(1)
(A)
(B)
(C)
Signal
(2)
(3)
(4)
Permeabilization and/or Internalization
N
M
ROS
Downstream signal transduction
V
(5)
Leakage of cell contents
= Plant defensin
= Membrane lipids
= Surface proteins
= Cellular proteins
▼ = Unidentified receptor
V = Vacuole
M = Mitochondria
N = Nucleus

Figure 3. Model explaining the possible steps involved in antifungal action of plant defensins. (1) (A) Plant defensins either bind to membrane lipids and/or surface proteins due to electrostatic or hydrophobic interaction(s) or (B) aggregate on the membrane due to electrostatic attraction thus causing mechanical pressure or (C) may transmit the signal through receptor across the membrane. (2) One of the above mentioned associations result in either permeabilization of the cell membrane and/or internalization of defensins by fungal cells. It is not clearly known if the interaction of plant defensins with fungal cell wall components and/or plasma membrane components is required for entry into the fungal cells. Internalized defensins or the signal may directly or indirectly interact with cellular molecules eventually affecting the downstream processes. (3) and (4) It is not clear yet if plant defensins have specific organelle and/or other cellular targets inside the fungal cell. A few plant defensins were shown to internalize into the cell organelles like nucleus and vacuole and others were shown to affect fungal mitochondria thus resulting in the production of ROS. (5) Higher concentrations of defensins may cause severe permeabilization of the membrane and hence leakage of the cell contents.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Plant defensins have been previously shown to induce rapid changes in ions fluxes in sensitive fungi. RsAFP2 and DmAMP1 caused rapid fluxes in *N. crassa* hyphae resulting in the Ca^{+2} uptake and K^{+} efflux and alkalization of incubation medium (*66*). In addition, a strong correlation between Ca^{+2} uptake and antifungal activity of RsAFP2 and its variants against *F. culmorum* was observed when high ionic strength medium was used in the antifungal assay (*46*). Interestingly, presence of extracellular Ca^{+2} significantly reduced the antifungal activity of MsDef1 against *F. graminearum* (*36*). Using mammalian cell lines, it was shown that MsDef1 but not RsAFP2 blocks the L-type calcium channel (*36*). In collaboration with Prof. Nick Read's lab at the University of Edinburgh, we have recently observed a significant increase in the intracellular calcium $[Ca^{+2}]_c$ levels in *N. crassa* treated with MsDef1 and MtDef4. Furthermore, both defensins altered the Ca^{+2} signatures generated in response to mechanical perturbation. Each defensin showed unique effects on the $[Ca^{+2}]_c$ amplitude and the $[Ca^{+2}]_c$ resting level (Muñoz et al., unpublished data). Maize defensins, γ-zeathionins were shown to rapidly inhibit the sodium channel blockage of GH3 tumor cell line (*35*). High structural similarity of zeathionins with insect and scorpion neurotoxins may explain their similar modes of action (*35*). However, blockage of Ca^{+2} channels by MsDef1 in a manner akin to virally encoded KP4 suggests their evolutionary convergence (*36*). Though intracellular ion concentrations and ion channels of sensitive organisms seem to be affected in the presence of plant defensins, more work needs to be done to clearly understand the effect of plant defensins on ion channels.

While the antifungal action of some plant defensins is clearly dependent on the presence of sphingolipids in the fungal plasma membrane, other defensins use completely different mechanisms for their antifungal action. Evidence is beginning to accumulate that some plant defensins are internalized by fungal cells and likely target intracellular components as part of their antifungal action. Internalization of plant defensins and the interaction with host intracellular components remained one of the major unanswered questions in their MOA. Using fluorophore-conjugated NaD1 and immunogold localization studies, *Nicotiana alata* defensin NaD1 has been shown to enter fungal cells (*62*). Recently, it was shown that entry of NaD1 into *F. oxysporum* f. sp. *vasinfectum* cells requires putative cell wall receptors (*67*). Our lab has recently determined that MtDef4 is also internalized by the cells of *F. graminearum* (Sagaram and Shah, unpublished data). Further studies are required to determine the molecular mechanisms for translocation of NaD1 and MtDef4 into fungal cells and to identify their intracellular targets. Only in case of pea defensin, PsD1, the intracellular target has been identified. Using the yeast 2-hybrid screening and pull down assays, PsD1 was shown to interact with *N. crassa* cyclin F protein involved in the cell cycle control. *In vivo* localization of PsD1 in *N. crassa* nuclei was shown using fluoresceine isothiocyanate (FITC)-conjugated PsD1 (*68*). Also, it is becoming increasingly evident that mitochondrial mediated production of reactive oxygen species (ROS) plays a critical role in antifungal action. Sensitive fungi challenged with NaD1 or RsAFP2 were shown to produce ROS (*62*, *69*). Recent study involving *C. albicans* showed that significant number of deletion mutants involved in mitochondrial functionality conferred at least 4-fold hypersensitivity

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

or resistance to *H. sanguinea* defensin HsAFP1, further suggesting mitochondrion as one of the targets of plant defensins (*70*). Apoptosis phenomenon followed by ROS production was also observed in the presence of RsAFP2 and HsAFP1 (*69*, *70*). In case of several other plant defensins, it still remains unclear what the exact mechanism of action is once the defensins are internalized. However, several possibilities have been proposed such as inhibition of cell wall synthesis, nucleic acid or protein synthesis, or enzymatic activity (*52*). Future studies will be helpful in identifying and characterizing specific intracellular targets or signal transduction pathways involved in the antifungal action by plant defensins. Based on current knowledge, a model explaining possible steps in antifungal action of plant defensins has been proposed in Figure 3. Finally, comprehensive knowledge about the complex mode of action of defensins will enhance our ability in rational design of *de novo* peptides with increased activity.

Expression of Antifungal Defensins in Transgenic Crops for Resistance to Fungal and Oomycete Pathogens

Since plant defensins are potent inhibitors of various plant pathogenic fungi, and are non-toxic to mammalian cells *in vitro* (*2*, *71*), many attempts have been made to engineer fungal resistant plants using plant defensins. While most of the studies reported that constitutive expression of plant defensins imparted enhanced resistance to various plant pathogens in greenhouse (*72–79*), so far only two studies have demonstrated fungal resistance under the field conditions (*40*, *80*). Several defensins discovered thus far have been shown to provide potent antifungal activity against a range of fungi *albeit* to varying degrees (Table I). Some plant defensins such as NmDef02 (*80*) and MtDef4 (J. Kaur, U. Sagaram and D. Shah, unpublished data) provide resistance to fungal and oomycete pathogens in transgenic plants (Table I). Additionally, as discussed earlier, it is becoming apparent that plant defensins employ different modes of antifungal action against target fungi and could potentially be deployed in transgenic plants to achieve durable resistance to a fungal pathogen. Considering aforementioned attributes, optimal spatial and temporal expression of plant defensins is the key to provide commercially acceptable levels of resistance to fungal pathogens in transgenic plants. However, real challenge here is achieving optimal expression of plant defensins in transgenic plants to match the unique lifestyle of a fungal or oomycete pathogen without any associated ill effects on the normal growth and development of the engineered plant. Almost all of the studies to date on engineering fungal resistance in plants have employed constitutive promoters to express defensins often resulting in the undesirable pleiotropic effects on the engineered plant's growth and development (*81*). Expressing antifungal defensin at the site of fungal attack using a pathogen-inducible promoter such as the barley *GER4c* promoter will be highly desirable. Promoter *GER4c* has been reported to be induced strongly and rapidly in response to fungal pathogens which exhibit biotrophic and necrotrophic lifestyles (*82*). One can also envisage using "designer promoters" for pathogen-inducible expression (*83*) or tissue-specific promoters for expression in tissues first colonized by the pathogen (*84*). Thus, in

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

case of a fungal pathogen *Ustilago maydis* which causes smut disease of maize, silk (stigma) channel has been suggested to be an important route of infection (*85*, *86*). Hence, using stigma specific promoter like *SLG* (*84*) would be a wise choice. Tissue-specific expression of defensins in transgenic crops could not only minimize any potential undesirable pleiotropic effects of transgene expression, but also permit avoiding expressing a protein in the edible tissues of the plant.

Based on their lifestyle, plant pathogens (both fungal and oomycete) can be divided into three different classes (*87*, *88*). Biotrophs (e.g., *H. arabidopsidis, Puccinia striiformis*) infect only living plant cells in order to complete their lifecycle. They grow in intercellular spaces or as intracellular haustoria bounded by the host plasma membrane. Hemibiotrophs (e.g, *F. graminearum*) have a brief biotrophic phase probably for initial establishment when they are restricted to the extracellular space in the host, before killing the host cells. On the other hand, necrotrophs (e.g., *Botrytis cinerea*) kill host cells to obtain nutrients for their growth and reproduction. The majority of plant defensins are secreted to the extracellular space and their extracellular localization might be sufficient to provide resistance to biotrophs which are restricted to the extracellular space during infection process. This is supported by our recent finding that expression of extracellularly targeted MtDef4 confers robust resistance to the obligate biotrophic oomycete *H. arabidopsidis* in transgenic *A. thaliana* plants, but MtDef4 targeted to vacuoles or retained in the endoplasmic reticulum does not (Kaur and Shah, unpublished data). In order to achieve effective control of hemibiotrophs or necrotrophs, coexpression of plant defensins in the extracellular space and in the intracellular organelles (targeted to vacuole or retained in endoplasmic reticulum) may be necessary (Kaur and Shah, unpublished data).

The co-expression of antifungal defensins and other antimicrobial proteins is also emerging as a promising approach for control of fungal pathogens in transgenic plants. In addition to defensins, plants produce several other classes of antimicrobial proteins including snakins, lipid-transfer proteins and osmotins (*89*). Plants also express a large number of pathogenesis-related proteins (PR1-PR17) some of which exhibit *in vitro* antifungal activity (*90*). Indeed, coexpression of antifungal defensins and other antimicrobial proteins in transgenic plants has been shown to provide better resistance to fungal pathogens than the expression of defensin genes alone (*91–93*).

In conclusion, plants produce a large number of defensins some of which exhibit potent *in vitro* antifungal activity against economically important fungal and oomycete pathogens. Rapid progress is being made in elucidating their structure-activity relationships and modes of antifungal action. Molecular tools are also available to facilitate spatial and temporal expression of these proteins in a manner consistent with the lifestyle of the pathogen. Our knowledge of plant defensins, combined with the availability of optimal gene expression tools, forms the basis for engineering robust and durable resistance to fungal and oomycete pathogens in transgenic crops.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

References

1. Strange, R. N.; Scott, P. R. *Annu. Rev. Phytopathol.* **2005**, *43*, 83–116.
2. Thomma, B. P. H. J.; Cammue, B. P. A.; Thevissen, K. *Planta* **2002**, *216*, 193–202.
3. Lay, F. T.; Anderson, M. A. *Curr. Protein Pept. Sci.* **2005**, *6*, 85–101.
4. Lehrer, R. I.; Ganz, T. *Curr. Opin. Immunol.* **2002**, *14*, 96–102.
5. Zasloff, M. *Nature* **2002**, *415*, 389–395.
6. Hancock, R. E.; Sahl, H. G. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.
7. Bloch, C., Jr.; Patel, S. U.; Baud, F.; Zvelebil, M. J.; Carr, M. D.; Sadler, P. J.; Thornton, J. M. *Proteins* **1998**, *32*, 334–349.
8. Fant, F.; Vranken, W.; Broekaert, W; Borremans, F. *J. Mol. Biol.* **1998**, *279*, 257–270.
9. Fant, F.; Vranken, W. F.; Borremans, F. A. *Proteins* **1999**, *37*, 388–403.
10. Almeida, M. S.; Cabral, K. M.; Kurtenbach, E.; Almeida, F. C.; Valente, A. P. *J. Mol. Biol.* **2002**, *315*, 749–757.
11. Janssen, B. J.; Schirra, H. J.; Lay, F. T.; Anderson, M. A.; Craik, D. J. *Biochemistry* **2003**, *42*, 8214–8222.
12. Lay, F. T.; Brugliera, F.; Anderson, M. A. *Plant Physiol.* **2003**, *131*, 1283–1293.
13. Lay, F. T.; Schirra, H. J.; Scanlon, M. J.; Anderson, M. A.; Craik, D. J. *J. Mol. Biol.* **2003**, *325*, 175–188.
14. Hanks, J. N.; Snyder, A. K.; Graham, M. A.; Shah, R. K.; Blaylock, L. A.; Harrison, M. J.; Shah, D. M. *Plant Mol. Biol.* **2005**, *58*, 385–399.
15. Sagaram, U. S.; Pandurangi, R.; Kaur, J.; Smith, T. J.; Shah, D. M. *PLoS One* **2011**, *6*, e18550.
16. Oomen, R. J.; Seveno-Carpentier, E.; Ricodeau, N.; Bournaud, C.; Conejero, G.; Paris, N.; Berthomieu, P.; Marques, L. *New Phytol.* **2011**, *192*, 140–150.
17. Epple, P.; Apel, K.; Bohlmann, H. *Plant Physiol.* **1995**, *109*, 813–20.
18. Brown, R. L.; Kazan, K.; McGrath, K. C.; Maclean, J. D.; Manners, J. M. *Plant Physiol.* **2003**, *132*, 1020–1032.
19. Koike, M.; Okamoto, T.; Tsuda, S.; Imai, R. *Biochem. Biophys. Res. Commun.* **2002**, *298*, 46–53.
20. Carvalho, A. O.; Filho, G. A.; Ferreira, B. S.; Branco, A. T.; Okorokova-Facanha, A. L.; Gomes, V. M. *Protein Pept. Lett.* **2006**, *13*, 1029–36.
21. Do, H. M.; Lee, S. C.; Jung, H. W.; Sohn, K. H.; Hwang, B. K. *Plant Sci.* **2004**, *166*, 1297–1305.
22. Mirouze, M.; Sels, J.; Richard, O.; Czernic, P.; Loubet, S.; Jacquier, A.; Francois, I. E.; Cammue, B. P.; Lebrun, M.; Berthomieu, P.; Marques, L. *Plant J.* **2006**, *47*, 329–342.
23. Meyer, B.; Houlne, G.; Pozueta-Romero, J.; Schantz, M. L.; Schantz, R. *Plant Physiol.* **1996**, *112*, 615–622.
24. Penninckx, I. A.; Eggermont, K.; Terras, F. R.; Thomma, B. P.; De Samblanx, G. W.; Buchala, A.; Metraux, J. P.; Manners, J. M.; Broekaert, W. F. *Plant Cell* **1996**, *8*, 2309–2323.
25. Epple, P.; Apel, K.; Bohlmann, H. *FEBS Lett.* **1997**, *400*, 168–172.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

26. Manners, J. M.; Penninckx, I. A.; Vermaere, K.; Kazan, K.; Brown, R. L.; Morgan, A.; Maclean, D. J.; Curtis, M. D.; Cammune, B. P.; Broekaert, W. F. *Plant Mol. Biol.* **1998**, *38*, 1071–1080.
27. Terras, F. R.; Penninckx, I. A.; Goderis, I. J.; Broekaert, W. F. *Planta* **1998**, *206*, 117–24.
28. Zimmerli, L.; Stein, M.; Lipka, V.; Schulze-Lefert, P.; Somerville, S. *Plant J.* **2004**, *40*, 633–646.
29. Zhang, Y.; Lewis, K. *FEMS Microbiol. Lett.* **1997**, *149*, 59–64.
30. Segura, A.; Moreno, M.; Molina, A.; Garcia-Olmedo, F. *FEBS Lett.* **1998**, *435*, 159–162.
31. Chen, G. H.; Hsu, M. P.; Tan, C. H. S.; Kuo, C. G. F.; Chen, H. M.; Chen, S.; Chen, C. S. *J. Agric. Food Chem.* **2005**, *53*, 982–988.
32. Aerts, A. M.; Francois, I. E.; Cammue, B. P.; Thevissen, K. *Cell Mol. Life Sci.* **2008**, *65*, 2069–2079.
33. Wijaya, R.; Neumann, G. M.; Condron, R.; Hughes, A. B.; Polya, G. M. *Plant Sci.* **2000**, *159*, 243–255.
34. Bloch, C., Jr.; Richardson, M. *FEBS Lett.*. **1991**, *279*, 101–104.
35. Kushmerick, C.; de Souza Castro, M.; Santos Cruz, J.; Bloch, C., Jr.; Bejrao, P. S. *FEBS Lett.* **1998**, *440*, 302–306.
36. Spelbrink, R. G.; Dilmac, N.; Allen, A.; Smith, T. J.; Shah, D. M.; Hockerman, G. H. *Plant Physiol.* **2004**, *135*, 2055–2067.
37. Amien, S.; Kliwer, I.; Marton, M. L.; Debener, T.; Geiger, D.; Becker, D.; Dresselhaus, T. *PLoS Biol.* **2010**, *8*, e1000388.
38. Carvalho, Ade O.; Gomes, V. M. *Peptides* **2009**, *30*, 1007–1020.
39. Stotz, H. U.; Thomson, J. G.; Wang, Y. *Plant Signal. Behav.* **2009**, *4*, 1010–1012.
40. Gao, A.; Hakimi, S. M.; Mittanck, C. A.; Wu, Y.; Woerner, M. B.; Stark, D. M.; Shah, D. M.; Liang, J.; Rommens, C. M. T. *Nat. Biotechnol.* **2000**, *18*, 1307–1310.
41. Ramamoorthy, V.; Zhao, X.; Snyder, A. K.; Xu, J. -R.; Shah, D. M. *Cell. Microbiol.* **2007**, *9*, 1491–1506.
42. Terras, F. R.; Schoofs, H. M.; De Bolle, M. F.; Van Leuven, F.; Rees, S. B.; Vanderleyden, J.; Cammue, B. P.; Broekaert, W. F. *J. Biol. Chem.* **1992**, *267*, 15301–15309.
43. Osborn, R. W.; De Samblanx, G. W.; Thevissen, K.; Goderis, I.; Torrekens, S.; Van Leuven, F.; Attenborough, S.; Rees, S. B.; Broekaert, W. F. *FEBS Lett.* **1995**, *368*, 257–262.
44. Yount, N. Y.; Yeaman, M. R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 7363–7368.
45. Ramamoorthy, V.; Cahoon, E. B.; Li, J.; Thokala, M.; Minto, R. E.; Shah, D. M. *Mol. Microbiol.* **2007**, *66*, 771–786.
46. De Samblanx, G. W.; Goderis, I. J.; Thevissen, K.; Raemaekers, R.; Fant, F.; Borremans, F.; Acland, D. P.; Osborn, R. W.; Patel, S.; Broekaert, W. F. *J. Biol. Chem.* **1997**, *272*, 1171–1179.
47. Wu, Z.; Hoover, D. M.; Yang, D.; Boulègue, C.; Santamaria, F.; Oppenheim, J. J.; Lubkowski, J.; Lu, W. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 8880–8885.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

48. Schroeder, BO.; Wu, Z.; Nuding, S.; Grosrurth, S.; Marcinowski, M.; Beisner, J.; Buchner, J.; Schaller, M.; Stange, EF.; Wehkamp, J. *Nature* **2011**, *469*, 419–423.
49. Maemoto, A.; Qu, X.; Rosengren, K. J.; Tanabe, H.; Henschen-Edman, A.; Craik, D. J.; Ouellette, A. J. *J. Biol. Chem.* **2004**, *279*, 44188–44196.
50. Thevissen, K.; Francois, I. E.; Aerts, A. M.; Cammue, B. P. *Curr. Drug Targets* **2005**, *6*, 923–928.
51. Thevissen, K.; Kristensen, H. H.; Thomma, B. P.; Cammue, B. P.; Francois, I. E. *Drug Discovery Today* **2007**, *12*, 966–971.
52. Brogden, K. A. *Nat. Rev. Microbiol.* **2005**, *3*, 238–250.
53. Ehrenstein, G.; Lecar, H. *Q. Rev. Biophys.* **1977**, *10*, 1–34.
54. Yang, L.; Harroun, T. A.; Weiss, T. M.; Ding, L.; Huang, H. W. *Biophys. J.* **2001**, *81*, 1475–1485.
55. Pouny, Y.; Rapaport, D.; Mor, A.; Nicolas, P.; Shai, Y. *Biochemistry* **1992**, *31*, 12416–12423.
56. Shai, Y. *Biochim. Biophys. Acta.* **1999**, *462*, 55–70.
57. Ladokhin, A. S.; White, S. H. *Biochim. Biophys. Acta.* **2001**, *1514*, 253–260.
58. Matsuzaki, K.; Murase, O.; Fujii, N.; Miyajima, K. *Biochemistry* **1996**, *35*, 11361–11368.
59. Yamaguchi, S.; Hong, T.; Waring, A.; Lehrer, R. I.; Hong, M. *Biochemistry* **2002**, *41*, 9852–9862.
60. Thevissen, K.; Osborn, R. W.; Acland, D. P.; Broekaert, W. F. *Mol. Plant-Microbe Interact.* **1997**, *13*, 54–61.
61. Thevissen, K.; Terras, F. R.; Broekaert, W. F. *Appl. Environ. Microbiol.* **1999**, *65*, 5451–5458.
62. van der Weerden, N. L.; Lay, F. T.; Anderson, M. A. *J. Biol. Chem.* **2008**, *283*, 14445–14452.
63. Thevissen, K.; Ferket, K. K.; Francois, I. E.; Cammue, B. P. *Peptides* **2003**, *24*, 1705–1712.
64. Thevissen, K.; Cammue, B. P.; Lemaire, K.; Winderickx, J.; Dickson, R. C.; Lester, R. L.; Ferket, K. K.; Van Even, F.; Parret, A. H.; Broekaert, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9531–9536.
65. Thevissen, K.; Warnecke, D. C.; Francois, I. E.; Leipelt, M.; Heinz, E.; Ott, C.; Zahringer, U.; Thomma, B. P.; Ferket, K. K.; Cammue, B. P. A. *J. Biol. Chem.* **2004**, *279*, 3900–3905.
66. Thevissen, K.; Ghazi, A.; De Samblanx, G. W.; Brownlee, C.; Osborn, R. W.; Broekaert, W. F. *J. Biol. Chem.* **1996**, *271*, 15018–15025.
67. van der Weerden, N. L.; Hancock, R. E.; Anderson, M. A. *J. Biol. Chem.* **2010**, *285*, 37513–37520.
68. Lobo, D. S.; Pereira, I. B.; Fragel-Madeira, L.; Medeiros, L. N.; Cabral, L. M.; Faria, J.; Bellio, M.; Campos, R. C.; Linden, R.; Kurtenbach, E. *Biochemistry* **2007**, *46*, 987–996.
69. Aerts, A. M.; Francois, I. E.; Meert, E. M.; Li, Q. T.; Cammue, B. P.; Thevissen, K. J. *Mol. Microbiol. Biotechnol.* **2007**, *13*, 243–247.
70. Aerts, A. M.; Bammens, L.; Govaert, G.; Carmona-Gutierrez, D.; Madeo, F.; Cammue, B. P. A.; Thevissen, K. *Front. Microbiol.* **2011**, *2*, 1–9.

71. Tavares, L. S.; Santos, Mde O.; Viccini, L. F.; Moreira, J. S.; Miller, R. N.; Franco, O. L *Peptides* **2008**, *29*, 1842–1851.
72. Terras, F. R.; Eggermont, K.; Kovaleva, V.; Raikhel, N. V.; Osborn, R. W.; Kester, A.; Rees, S. B.; Torrekens, S.; Van Leuven, F.; Vanderleyden, J. *Plant Cell* **1995**, *7*, 573–588.
73. Wang, Y. P.; Nowak, G.; Culley, D.; Hadwiger, L. A.; Fristensky, B. *Mol. Plant-Microbe Interact.* **1999**, *12*, 410–418.
74. Parashina, E. V.; Serdobinskii, L. A.; Kalle, E. G.; Lavorova, N. V.; Avetisov, V. A.; Lumin, V. G.; Naroditski, B. S. *Russ. J. Plant Physiol.* **2000**, *47*, 417–423.
75. Kanzaki, H.; Nirasawa, S.; Saitoh, H.; Ito, M.; Nishihara, M.; Terauchi, R.; Nakamura, I. *Theor. Appl. Genet.* **2002**, *105*, 809–814.
76. Peschen, D.; Li, H. P.; Fischer, R.; Kreuzaler, F.; Liao, Y. C. *Nat. Biotechnol* **2004**, *22*, 732–738.
77. Schaefer, S. C.; Gasic, K.; Cammue, B.; Broekaert, W.; van Damme, E. J.; Peumans, W. J.; Korban, S. S. *Planta* **2005**, *222*, 858–866.
78. Jha, S.; Chattoo, B. B. *Transgenic Res.* **2009**, *19*, 373–384.
79. De Coninck, B. M.; Sels, J.; Venmans, E.; Thys, W.; Goderis, I. J.; Carron, D.; Delaure, S. L.; Cammue, B. P.; De Bolle, M. F.; Mathys, J. *New Phytol.* **2010**, *187*, 1075–1088.
80. Portieles, R.; Ayra, C.; Gonzalez, E.; Gallo, A.; Rodriguez, R.; Chacon, O.; Lopez, Y.; Rodriguez, M.; Castillo, J.; Pujol, M.; Enriquez, G.; Borroto, C.; Trujillo, L.; Thomma, B. P.; Borras-Hidalgo, O. *Plant Biotechnol. J.* **2010**, *8*, 678–690.
81. Kaur, J.; Sagaram, U. S.; Shah, D. M. *Fungal Biol. Rev.* **2011**, *25*, 128–135.
82. Himmelbach, A.; Liu, L; Zierold, U.; Altschmied, L.; Maucher, H.; Beier, F.; Muller, D.; Hensel, G.; Heise, A.; Schutzendubel, A.; Kumlehn, J.; Schweizer, P. *Plant Cell* **2010**, *22*, 937–952.
83. Rushton, P. J.; Reinstadler, A.; Lipka, V.; Lippok, B.; Somssich, I. E. *Plant Cell* **2002**, *14*, 749–762.
84. Liu, Z.; Changhe, Z.; Wu, K. *Plant Cell Rep.* **2008**, *27*, 995–1004.
85. Christensen, J. J. *The American Phytopathological Society*; St. Paul, MN, 1963.
86. Snetselaar, K. M.; Carfioli, M. A.; Cordisco, K. M. *Can. J. Bot.* **2001**, *79*, 1390–1399.
87. Oliver, R. P.; Ipcho, S. V. *Mol. Plant. Pathol.* **2004**, *5*, 347–352.
88. Glazebrook, J. *Annu. Rev. Phytopathol.* **2005**, *43*, 205–227.
89. Garcia Olmedo, F.; Molina, A.; Alamillo, J. M.; Rodriguez Palenzuela, P. *Biopolymers* **1998**, *47*, 479–491.
90. van Loon, L. C.; Rep, M.; Pieterse, C. M. *Annu. Rev. Phytopathol.* **2006**, *44*, 135–162.
91. Oh, B. J.; Ko, M. K.; Kostenyuk, I; Shin, B.; Kim, K. S. *Plant Mol. Biol.* **1999**, *41*, 313–319.
92. Chen, S. C.; Liu, A. R.; Zou, Z. R. *Plant Physiol Russ J* **2006**, *53*, 671–677.
93. Ntui, V. O.; Azadi, P; Thirukkumaran, R. S.; Chin, D. P.; Nakamura, I.; Mii, M. *Plant Pathol* **2011**, *60*, 221–231.
94. Olli, S.; Kirti, P. B. *J. Biochem. Mol. Biol.* **2006**, *39*, 278–283.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

95. De Beer, A.; Vivier, M. A. *BMC Plant Biol.* **2008**, *8*, 75.
96. Finkina, E. I.; Shramova, E. I.; Tagaev, A. A.; Ovchinnikova, T. V. *Biochem. Biophys. Res. Comm.* **2008**, *371*, 860–865.
97. Odintsova, T. I.; Rogozhin, E. A.; Baranov, Y.; Musolyamov, A. Kh.; Yalpani, N.; Egorov, T. A.; Grishin, E. V. *Biochemie* **2008**, *90*, 1667–1673.
98. Ye, X. Y.; Ng, T. B. *J. Peptide Res.* **2002**, *60*, 81–87.
99. Park, H. C.; Kang, Y. H.; Chun, H. J.; Koo, J. C.; Cheong, Y. H.; Kim, C. Y.; Kim, M. C.; Chung, W. S.; Kim, J. C.; Yoo, J. H.; Koo, Y. D.; Koo, S. C.; Lim, C. O.; Lee, S. Y.; Cho, M. J. *Plant Mol. Biol.* **2002**, *50*, 59–69.
100. Wisniewskia, M. E.; Bassetta, C. L.; Artlipa, T. S.; Webba, R. P.; Janisiewicza, W. J.; Norellia, J. L.; Goldwayb, M.; Droby, S. *Physiol. Plant.* **2003**, *119*, 563–572.
101. Chen, G. H.; Hsu, M. P.; Tan, C. H.; Sung, H. Y.; Kuo, C. G.; Fan, M. J.; Chen, H. M.; Chen, S.; Chen, C. S. *J. Agric. Food Chem.* **2005**, *53*, 982–988.
102. Turrini, A.; Sbrana, C.; Pitto, L.; Castiglione, M. R.; Giorgetti, L.; Briganti, R.; Bracci, T.; Evangelista, M.; Nuti, M. P.; Giovannetti, M. *New Phytol* **.2004**, *163*, 393–403.
103. Wong, J. H.; Ng, T. B. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 1626–1632.
104. Wang, H. X.; Ng, T. B. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 576–581.
105. Kant, P.; Liu, W. Z.; Pauls, K. P. *Peptides* **2009**, *30*, 1593–1599.
106. Games, P. D.; Santos, I. S. dos.; Mello, O. E.; Diz, M. S. S.; Carvalho, A. O.; de Souza Filho, G. A.; Cunha, M. D.; Vasconcelos, I. M.; Ferreira, B. S.; Gomes, V. M. *Peptides* **2008**, *29*, 2090–2100.
107. Solis, J.; Medrano, G.; Ghislain, M. *J. Plant Physiol.* **2007**, *164*, 1071–1082.
108. Zainal, Z.; Marouf, E.; Ismail, I.; Fei, C. K. *Amer. J. Plant Physiol.* **2009**, *4*, 70–79.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch015

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Chapter 16

Antifungal Peptides: Exploiting Non-Lytic Mechanisms and Cell Penetration Properties

**Jose F. Marcos,*,[1] Mónica Gandía,[1] Eleonora Harries,[1]
Lourdes Carmona,[1] and Alberto Muñoz[2]**

**[1]Instituto de Agroquímica y Tecnología de Alimentos (IATA)-CSIC, Avda.
Agustín Escardino–7, 46980 Paterna, Valencia, Spain
[2]Fungal Cell Biology Group, Institute of Cell Biology, University of
Edinburgh, Rutherford Building, Edinburgh EH9 3JH, U.K.
*E-mail: jmarcos@iata.csic.es**

Antimicrobial peptides with antifungal activity have not received as much consideration as their antibacterial counterparts, which is probably a consequence of the higher impact of bacterial infections in human health. However, the emergence of mycoses as consequence of modern medical therapies on one hand, and the urgent need to find alternatives to fungicide use in agricultural and food industries, on the other, have increased the interest in antifungal peptides. Non-lytic modes of action have been reported in an array of antifungal peptides from diverse origins, both natural and synthetic, and are linked to low toxicity against non-target organisms, a desirable property for antimicrobial drugs. These non-lytic mechanisms include non-disruptive cell internalization, similar to cell penetrating peptides. Once inside the fungal cell, killing may occur by interference with various cell processes. Current knowledge of these mechanisms will be reviewed as well as the impact on the design of novel peptides and the identification of new antifungal targets.

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Introduction

Antimicrobial peptides (AMP) and peptide-related molecules are widespread in nature in organisms all along the phylogenetic scale (*1*, *2*). They include peptides and small proteins that have direct killing or inhibitory activity against microorganisms. As reviewed extensively in this book, AMP have been proposed as novel antimicrobials in view of the decreasing efficacy of conventional antibiotics in clinic (*2*, *3*), as alternatives to the massive use of fungicides in plant protection (*4*, *5*), or as food preservatives (*6*, *7*).

Broad spectrum activity of AMP expands in some cases not only to Gram positive and negative bacteria but also to fungi. Such a wide activity is often associated to undesirable toxicity against non-target organisms including humans, animals and plants, this being a negative characteristic that the rational design of peptides aims to counteract (*4*). Cytotoxicity is frequently due to interaction and disruption of biological membranes followed by cell lysis, which occurs because of the amphipathic and cationic properties of many AMP. However, as we will discuss in detail, cell disruption is not the only antimicrobial mechanism for an increasing number of AMP that also affect other cellular functions, and are therefore considered as multi-target drugs. Multiple mechanism of action within a single molecule is a desirable property that would make more difficult the selection of microbial resistance. Moreover, these “non-lytic” modes of action are presumably linked to increased specificity for microbes and thus lower non-specific toxicity to animal and plant cells.

Most of the effort in the identification and study of AMP has been dedicated to their antibacterial activity, as a reflection of the importance of their potential application to fight human diseases (*8*). However, this chapter will focus on antifungal rather than antibacterial peptides. The impact of fungi onto human health is qualitatively and quantitatively lower than that of bacterial infections, although in recent years interest has expanded due to increased resistance of fungal pathogens to current antifungal drugs and the practice of immunosuppression therapies that increase sensitivity to otherwise nonpathogenic fungi. The scenario in agriculture is different. Most of the infections that affect crop production and hence food supply are caused by fungi. Therefore, enormous resources are dedicated to fight and control such fungal plant infections, including a very large amount of fungicides applied in the field or during postharvest conservation, with negative impact on human and plant health and the environment. These reflections are on the basis of the increased attention shifted in the latter years to the identification, characterization and use of antifungal peptides.

There are a number of reports describing antifungal peptides that primarily do not affect cell permeability and are thus non-cytolytic. The characterization of the mode of action and identification of their targets is of top priority if peptides are to be considered as antifungals of practical use. This information is also critical to develop their mode of application/delivery. Finally, mechanistic knowledge should aid in the (rational) design of more powerful and specific peptides –a task for which peptides are particularly suitable- and even illuminate novel fungal targets for therapy.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

This chapter will summarize the properties of a selection of peptides/proteins that have been characterized as having non-lytic modes of antifungal action, and discuss the current knowledge and future research on these mechanisms.

Antifungal Peptides

There are an increasing number of peptides, both natural and synthetic, which primarily exhibit antifungal activity. The advantage of peptides with increased specificity for fungi is that the therapeutic potential is greater than that of peptides with broad spectrum antimicrobial activity. As occurs broadly in AMP, these peptides and proteins vary in length and amino acid composition, but most of them are cationic and amphipathic (Figure 1).

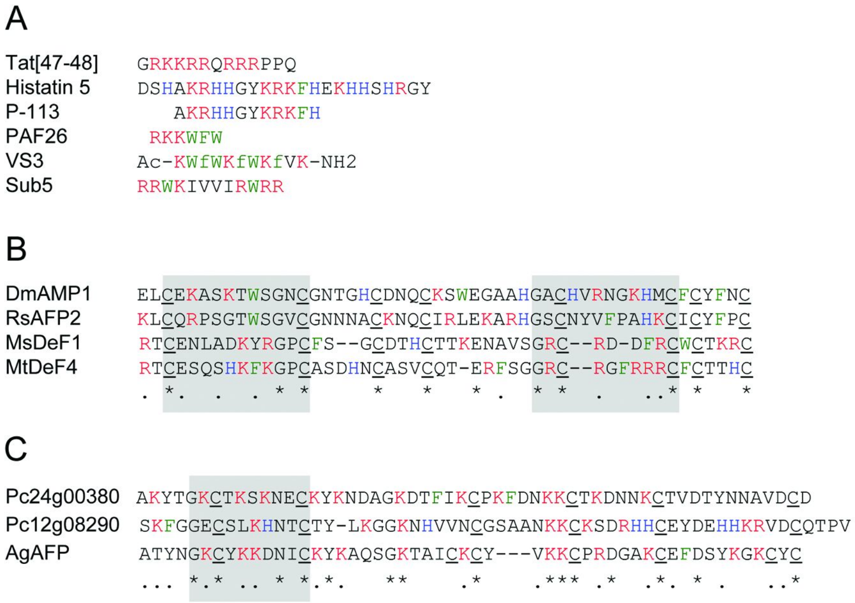

A
Tat[47-48] GRKKRRQRRRPPQ
Histatin 5 DSHAKRHHGYKRKFHEKHHSHRGY
P-113 AKRHHGYKRKFH
PAF26 RKKWFW
VS3 Ac-KWfWKfWKfVK-NH2
Sub5 RRWKIVVIRWRR
B
DmAMP1 ELCEKASKTWSGNCGNTGHCDNQCKSWEGAAHGACHVRNGKHMCFCYFNC
RsAFP2 KLCQRPSGTWSGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC
MsDeF1 RTCENLADKYRGPCFS--GCDTHCTTKENAVSGRC--RD-DFRCWCTKRC
MtDeF4 RTCESQSHKFKGPCASDHNCASVCQT-ERFSGGRC--RGFRRRCFCTTHC
. *. . . * * * * * . * * . ..* * *
C
Pc24g00380 AKYTGKCTKSKNECKYKNDAGKDTFIKCPKFDNKKCTKDNNKCTVDTYNNAVDCD
Pc12g08290 SKFGGECSLKHNTCTY-LKGGKNHVVNCGSAANKKCKSDRHHCEYDEHHKRVDCQTPV
AgAFP ATYNGKCYKKDNICKYKAQSGKTAICKCY---VKKCPRDGAKCEFDSYKGKCYC
... *.* .. * *.*. ** .* .*** .* .*. * . ..*

Figure 1. Amino acid sequence of antifungal peptides described in the text. (A) Linear antifungal peptides with cell penetrating activity. (B) Sequence alignment of the plant defensins DmAMP1, RsAFP2, MsDef1 and MtDef4 (21, 78). (C) Sequence alignment of the antifungal proteins identified in the genome of P. chrysogenum (Pc24g00380 and Pc12g08290) orthologous to PAF (38, 90), and of AFP from A. giganteus (AgAFP) (31). Residues are color coded to emphasize peptide properties as follows: cationic residues in red, aromatic residues in green, and histidines in blue. The unnatural residue α,β-dehydrophenylalanine from peptide VS3 is noted in lower case. The antimicrobial γ-core motif (14) is boxed in grey. (see color insert)

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Several remarkable examples of natural peptides and proteins show high activity towards fungal microbes. Cecropins are a class of antimicrobial peptides isolated first from the hemolymph of insects (*9*, *10*). They have broad spectrum antimicrobial activity against bacteria and fungi. Cecropins range from 29 to 42 amino acids in length and form amphipathic α-helices in hydrophobic environments such as the plasma membrane. Although the primary target of cecropins has been described as the plasma membrane, cecropin A at its microbicidal dose does not affect mammalian cells and studies have shown that this peptide can be administered safely to animals (*11*). Cecropin A effectively killed fungi such as *Aspergillus* spp. and *Fusarium* spp. (*10*). Importantly, cecropin synthetic analogs and hybrid sequences fused to other peptides have been designed to improve the antifungal properties (*12*, *13*).

An important number of natural AMP are rich in cysteine residues and contain disulfide bonds that stabilize their structure, which includes the γ-core motif (GXCX3-9C) as a signature common to these antimicrobial proteins (*14*) (see also Figure 1). Defensins are small disulfide-stabilized antimicrobial proteins (*2*, *15–17*). They are a large family of related but diverse proteins that have been found in mammals, insects, plants, and fungi. Mammalian defensins contain six conserved cysteine residues that form three disulfide bridges and are divided into three subfamilies: α- and β-defensins have been found in many mammalian species while θ-defensins only in *Rhesus* macaques. In addition to a direct antimicrobial activity, mammalian defensins also have other properties related to defense, such as chemotactic activity for immune system cells, pro-inflammatory signaling, or binding to membrane glycoproteins. Whereas most mammalian defensins have been shown active against *Candida albicans*, there are significant variations in their activities against other fungi. Recent findings show an interesting property of human α-defensin 1; the antifungal activity is increased after reduction of disulphide bridges due to a conformational switch, which unmask antimicrobial motifs and provides an additional level of regulation (*18*).

Plant defensins present molecular masses around 5 kDa and possess a common pattern of eight cysteine residues. They are structurally related to insect defensins and have several biological activities related to plant defense (*15*, *19*, *20*). Members of the plant defensin family have also enzyme (α-amylase or trypsin) or ion channel inhibitory activities. Plant defensins are best known for inhibiting the growth of a broad range of fungi, including plant and human pathogens, at micromolar concentrations as is the case for RsAFP1 and RsAFP2 from *Raphanus sativus*, MsDef1, MtDef2 and MtDef4 from *Medicago* spp., Dm-AMP1 and Dm-AMP2 from *Dahlia merckii*, Psd1 from *Pisum sativum* or NaD1 from *Nicotiana alata* (*19*, *21*, *22*) (see examples in Figure 1B). Resistance to fungal pathogens in plants transformed with defensin genes has been demonstrated (*23*, *24*). In plants, there are also antifungal proteins among the broad class of so-called pathogenesis-related proteins (PR). For instance, the tobacco osmotin belongs to the PR-5 class and has been shown to have strong and specific antifungal activity (*25*).

The antimicrobial protein plectasin isolated from the saprophytic fungus *Pseudoplectania nigrella* can also be ascribed to the class of defensins, and has an obvious biotechnological potential due to the ease of recombinant production

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

(*26*, *27*). Other fungi also produce and secrete small cysteine-rich antifungal proteins (Figure 1C). Examples include the antifungal protein AFP secreted by the imperfect ascomycete *Aspergillus giganteus* (*28–30*), which efficiently inhibits the growth of other filamentous fungi, including a variety of serious human and plant pathogens mainly of the genera *Aspergillus* and *Fusarium*, whereas it does not affect the viability of yeast, bacterial, plant and mammalian cells (*31*). Other members of the same class of AMP are those from *Penicillium chrysogenum* (*32*, *33*), *Aspergillus niger* (*34*), *Penicillium nalgiovense* (*35*), *Neosartorya fischeri* (*36*) and *Aspergillus clavatus* (*37*). Although these antifungal proteins are closely related from a structural point of view, they show slightly different functionality and antifungal specificity (*38*). Similar to plectasin, a very important aspect of AFP and related proteins is their biotechnological production through heterologous expression in bacteria or yeast (*39*, *40*). Interestingly, the biological role(s) that this class of "fungal" antifungal proteins fulfills in the natural producer fungus is still obscure. Future research should aim to shed light on the functions of these proteins that, at least in the case of *P. chrysogenum* seem to be represented by more than one protein/gene.

Histatins are a distinct group of linear cationic peptides that are isolated from human saliva and have potent and specific biological activity against fungi. Their high content in the amino acid histidine, weak amphipathic character and lack of disulfide bonds, distinguishes them from other cationic peptides (*41*). A key feature of histatins is their strong candidacidal activity (*42*), but they are also active against *Cryptococcus neoformans* and *Aspergillus fumigatus* (*43*). Histatin 5 has the strongest fungicidal activity against *C. albicans* (*42*), but it is also very active against phytopathogenic fungi (Marcos *et al.*, unpublished observations) (Figure 1A). P-113, a 12-mer amino acid fragment of histatin 5, is the smallest peptide that retains full anticandidal activity compared to its parental peptide (*44*) (Figure 1A). This is a first example on how synthetic peptides can aid not only in the identification of minimum antimicrobial motifs, but also in the design of novel antifungal sequences and characterization of mode of action (see below).

Other examples of synthetic non-natural antifungal peptides include the rationally designed D4E1 that shows antifungal properties consistent with its binding to ergosterol, a sterol characteristic of fungal membranes (*45*).

Nowadays, most of the experiments to identify AMP assay for *in vitro* growth inhibition of selected microorganisms, and are carried out using high throughput screenings that use microtiter plates. In our experience, assays for specific activity towards fungi (as opposed to bacteria) select for peptides with reduced non-specific toxicity or membrane disrupting properties, which presumably have non-lytic modes of action. A clear example is the synthetic hexapeptide PAF26 that has high activity (low micromolar range) towards filamentous fungi, and was selected in a combinatorial screening against the phytopathogen *Penicillium digitatum* while discarding peptides showing similar high activity against either *Saccharomyces cerevisiae* or the bacteria *Escherichia coli* (*46*) (Figure 1A). The activity of PAF26 against these latter is reduced; for instance the peptide concentration inhibitory to *S. cerevisiae* is ten-fold higher than to filamentous fungi. The hemolytic activity of PAF26 is non-detectable, as opposed to other short AMP.

A great interest due to their potential clinical applications has risen from β-1,3-glucan synthesis inhibitors such as echinocandins (*47*, *48*). These specific fungal inhibitors are cyclic lipopeptides that non-competitively inhibit the membrane-inserted β-glucan synthase (FKS1p), an enzyme critical for fungal cell wall biosynthesis and integrity. Echinocandin B is produced by *A. nidulans* and *A. rugulosus* (*49*) and was found to have potent antifungal activity against *Candida* spp. Nevertheless, it was an unsuitable antifungal candidate because it induced lysis of red blood cells. Analogs with improved efficacy and safety profile and greater aqueous solubility have now been developed and are on the market (*48*).

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Non-Lytic Modes of Antifungal Action

The mode of antimicrobial action of AMP was initially related to the capacity of cationic amphipathic peptides to interact and disrupt biological membranes, thus resulting in direct killing through cell permeabilization and lysis. Treatment of microorganisms with above minimal inhibitory AMP concentrations results in cell permeabilization that correlates with microbicidal potency. As consequence, many of the contributions that dealt with AMP mechanisms relied on models of peptide disruption of biological membranes. Although this holds true for most AMP, it is becoming increasingly evident that alternative mechanisms exist, even among peptides known as membrane disrupting (*50–54*). Significant examples reproduced in more than one AMP include the binding to membrane components (*55–57*), the interaction with chaperone like proteins (*58*, *59*), or the induction of DNA damage and apoptosis (*60–62*) or reactive oxygen species (*63–65*). As result, some AMP are viewed as multi-target drugs with overlapping actions that should ameliorate the increase of microbial resistance (*66*). Although this list includes both antibacterial and antifungal peptides, several of these examples of alternative or non-lytic modes of action are related to fungi and some specific cases have been only characterized in fungal microbes.

In addition, treatment with distinct AMP either at inhibitory (*67–69*) or sub-inhibitory concentrations (*70*) induced specific transcriptomic changes not primarily related to cell lysis. Global annotation of these induced changes points to the involvement of specific intracellular processes (see below). Although indirect, these observations are also consistent with a non-lytic mode of action.

It is difficult to depict a general scheme for the mechanism of action of this class of antifungal peptides. However, it is clear that the first step in their action must be the interaction with the outer cell envelopes (cell wall and cell membrane).

Interaction of AMP with Fungi

Peptides must first interact with the target fungus in order to exert their antimicrobial activity. Therefore, the relevance of fungal cell wall should not be underestimated as first interaction layer with antifungal peptides. Cell wall (CW) is an important structure in fungi that protects the cell against physical damage, maintains its shape, is essential to maintain stable osmotic conditions inside the cell and regulates biochemical exchange with the environment (*71*).

The biosynthesis, composition and structure of the fungal CW are unique to fungi and therefore have long been considered a promising target for the development of antifungal drugs (*72*, *73*), as exemplified in the case of echinocandins (see above). Fungal CW consists of a complex matrix of interlinked glycans and proteins, as chitin (a glycan polymer of N-acetylglucosamine), glucans (mostly β-1,3-glucan but also other such as β-1,6-glucan), mannans and glycoproteins. Proteins comprise a very significant amount of dry weight in the cell wall (from 15 up to 50%), they are densely decorated with both N- and O-linked carbohydrates, and many of them also contain a glycosylphosphatidylinositol (GPI) anchor. The overall net charge of fungal cell wall is negative under physiological conditions which explain the electrostatic attraction of cationic AMP.

Numerous studies have reported morphological alterations in fungal cells exposed to AMP (*12*, *74–77*). Morphological changes induced by antifungal peptides include changes in cell shape such as thickening of CW, cell enlargement to resemble balloon-like structures, or increase of staining with the chitin-binding fluorophore calcofluor white (CFW). Changes in the growth pattern of hyphae are also observed, including hyperbranching or dichotomous tip branching. These responses can be related to the stress the cell is sensing and are indicative of the importance of CW in the interaction with peptides but also as a defense response to counteract peptide action.

Plant defensins, for instance, have been classified according to the production of morphological changes in sensitive fungi (*20*, *21*). The major determinants of the antifungal activity and morphogenicity in the related *Medicago* defensins MsDef1 and MtDef4 have recently been found in their γ-core motifs (*78*) (Figure 1B). The change CRDDFRC to CRGFRRRC within the second γ-core motif of MsDef1 increased the activity and abolished its effects on morphology, mimicking the properties of the MtDef4 donor. Synthetic peptides that included this sequence also displayed a very significant antifungal activity against *F. graminearum*.

Thus, two maybe overlapping roles can be predicted for fungal CW in the interaction with peptides: as an interacting structure but also as a protective barrier. In the protective role, specific CW PIR proteins confer resistance to the plant antifungal osmotin in a hypersensitive *S. cerevisiae* strain, and deletion of the corresponding genes resulted in hypersensitivity of otherwise osmotin-tolerant common strains (*79*). Yeast spheroplasts devoid of CW obtained by enzymatic degradation from tolerant or sensitive strains were equally sensitive to osmotin. Another recognized example is that of the antibacterial peptide nisin, which is not active against yeast or filamentous fungi; however, yeast spheroplasts are lysed in the presence of nisin at concentrations which do not affect intact cells (*80*). Deletions of *CWP1/2* genes coding for CW mannoproteins increased sensitivity of yeast to nisin. On the other hand, a transcriptomic approach highlighted that the induction of genes coding for CW proteins is a response common after exposure of yeast to AMP of different modes of action, either cell penetrating (see below) or even cytolytic (*70*). However, in this case gene deletion of a subset of these induced CW-related genes did not lead to significant differences in terms of sensitivity to peptides, which was most likely due to gene redundancy.

There are examples in which the presence of the CW (or specific CW components) is required for the antifungal activity of the peptide. The plant

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

defensin NaD1 requires an intact cell wall since, contrarily to the above mentioned examples, enzymatic degradation with either proteinase K or w-glucanase prevented killing of *Fusarium* cells (*81*). More specifically, the CW protein SSA1/2p mediates cell wall binding and activity of the AMP histatin 5 against *Candida* (*82*), although cell wall β-glucans also contribute to binding (*83*). The involvement of SSA1/2 protein seems to expands to other AMP such as human β-defensins (*84*), in a situation analogous to the bacterial lipid II, which is a docking moiety for distinct classes of AMP such as bacterial nisin and fungal plectasin (*55*, *56*). SSA1/2p is a protein chaperone that belongs to the stress-related heat shock protein 70 (HSP70) family and locates not only in the cell wall but also in the cytoplasm and vacuolar membranes. A subsequent study mapped the histatin 5-binding epitope to the ATP binding domain of SSA2p (*59*).

Many of these CW proteins are heavily glycosylated. Not surprisingly glycosylation has a significant role in determining sensitivity to AMP. Different studies have reported that genes coding for components of mannosyltransferases that N-links phosphomannans to yeast CW mannoproteins facilitate toxicity of antifungal proteins and synthetic peptides, and they do so by promoting binding to CW (*85–87*). Conversely, deletion of these genes results in loss of mannosyl phosphate in the CW and increased resistance. These studies do not clarify whether CW phosphomannans serve as binding motifs to AMP or just confer the negative charge that attracts AMP to cells.

Carbohydrate-binding protein motifs are widespread in nature and are also found in antifungal proteins (*40*, *88*, *89*). The AFP from *A. giganteus* was reported to bind chitin, inhibit chitin biosynthesis and cause CW stress in sensitive fungi (*89*). However, chitin binding was shown to be independent of inhibitory activity in *F. oxysporum* and *Aspergillus* spp. Mutants of *F. oxysporum* and *A. oryzae* in chitin synthase genes of classes III and V were shown to have increased resistance to this antifungal protein. An independent study showed partially conflicting data regarding the sensitivity of different chitin synthase mutants of *F. oxysporum*, but also that binding to AFP does not correlate with sensitivity of these mutants to the protein (*90*), thus confirming that chitin binding may be necessary but does not explain inhibitory activity. The related *P. chrysogenum* PAF exhibits no chitobiose binding affinity despite the presence of a conserved chitin binding motif, which also includes a lysine residue important for the toxicity of the protein (*40*). *S. cerevisiae* responds to AFP with increased chitin synthesis thus indicating the protecting role of chitin, and deletion of specific chitin synthase genes resulted in increased sensitivity to AFP (*91*).

CW is not the unique interaction layer. Cell membrane glycolipids have also been related to the activity of antifungal peptides and proteins (*22*). The plant defensin RsAFP is able to bind the neutral sphingolipid class of glucosylceramides (GlcCer) of fungal membranes, and yeast mutants deficient in the corresponding biosynthetic gene *GCS1* are resistant to RsAFP2 (*57*). Similarly, the genes *IPT1* and *SKN1* involved in the biosynthesis of the acid sphingolipid mannosyl di-inositol phosphoryl-ceramide, determine the sensitivity of yeast to the plant defensin DmAMP1 (*92*, *93*). This is a remarkable example since deletion of *IPT1* results in enhanced resistance not only to related plant defensins, but also to other completely unrelated antifungal peptides such as the cyclic lipopeptide syringomycin E (*94*) or the synthetic hexapeptide PAF26 (*70*). Therefore, a "binding" function (as initially assumed) for this class of lipids might not be the (sole) explanation for the observed phenotype. Findings that point to the involvement of sphingolipids in apoptosis, response to oxidative stress, or autophagy in fungi (*95*, *96*), could indicate a role in the intracellular cell response/signaling to peptide exposure (see below). But in any case, there is a degree of specificity, since the homologous *F. graminearum GCS1* gene involved in the biosynthesis of GlcCer mediates the susceptibility to the plant MsDef1 defensin but, interestingly, not to the related MtDef4 (*97*).

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Cell Penetration Properties of Antifungal Peptides

Once access is gained to cell membrane through diffusion along the cell wall, membrane disruption and permeation are still considered a major property and determinant of activity of most AMP. However, cell penetrating properties are being demonstrated for an increasing number of peptides. These are able to cross biological membranes without membrane permeation and translocate into the cell where presumably their antimicrobial activity takes place. Insect apidaecin, a proline-rich AMP was one of the first AMP for which a translocation and non-pore forming mechanism against bacteria was invoked (*98*). In the case of the amphibian buforin II, a proline residue was found determinant for the intracellular location in *E. coli* and also for increased activity against other bacteria and fungi (*99*). Both peptides belong to the proline-rich class of AMP, which are able to penetrate into susceptible bacteria where they then act intracellularly (*100*). However, cell penetration is also exerted by some antifungal peptides.

Most AMP show strong similarities in biophysical properties with the so-called cell-penetrating peptides (CPP). CPP enter eukaryotic cells in a non-disruptive manner without apparent cytotoxicity (*101*, *102*). Currently, there is a number of AMP that have been convincingly demonstrated to enter microbial cells at low to moderate concentrations (see below). Conversely, antimicrobial and microbe-penetrating activities have been demonstrated in CPP such as Tat[47-58] derived from the HIV-1 regulatory Tat protein shown to be antifungal against *Candida* (*103*). Not surprisingly, the discussion is open regarding the extent at which cell penetration is related to antimicrobial activity or just a rare property of a restricted class of AMP (*53*, *104–107*). AMP with microbe penetration properties also tend to be among the most species-specific.

The underlying mechanisms of cell penetration by CPPs involve two general pathways: direct translocation and endocytosis (*108*). Currently, it is thought that both mechanisms can occur simultaneously and that the balance between them depends on peptide concentration, cell factors, and binding of CPP to specific cell membrane components (*109*, *110*). Even different endocytic pathways might contribute simultaneously to the cell penetration of selected peptides (*111*, *112*).

It is logical to hypothesize that CPP can interfere with intracellular processes and cell homeostasis in different ways (*113*), resulting in growth alteration/inhibition and thus antimicrobial activity. Examples of cell penetration properties can be found in several antifungal peptides. The use of fluorescently

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

labeled peptides/proteins in combination with confocal microscopy has demonstrated cell internalization (Figure 2). Distinct fungal antifungal proteins have been shown to be located intracellularly in sensitive fungal cells (*114*, *115*). *A. giganteus* AFP even localizes in the cell nucleus of the rice blast fungus *Magnaporthe oryzae* (*114*). The plant defensin Psd1 also showed *in vivo* co-localization with DAPI-stained fungal nuclei in the model fungus *Neurospora crassa* (*116*). Apparently none of these two proteins have canonical nuclear location signals. On the contrary, the *P. chrysogenum* PAF localizes to the cytoplasm of sensitive aspergilli, but does not colocalize with nuclei (*40*, *115*). Another plant defensin, NaD1, has been recently demonstrated to enter the cytoplasm of *F. oxysporum* hyphae (*117*). NaD1 bound to the CW of treated hyphae, and entered into a subpopulation of cells resulting in granulation of the cytoplasm and cell death. These results suggest that the activity of these antifungal proteins may not be restricted to the fungal cell membrane and that they may affect intracellular targets. However, in all these examples, the translocation mechanism remains largely unclear.

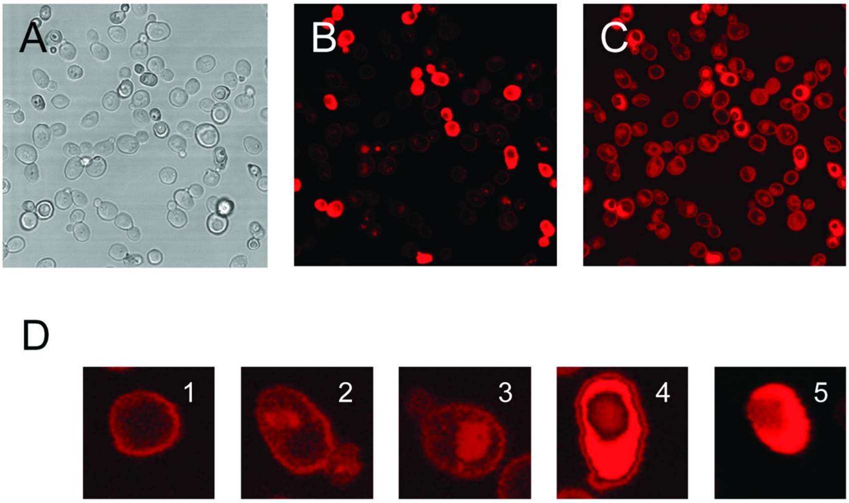


Figure 2. Localization of fluorescently labeled PAF26 in S. cerevisiae BY4741 (1 x 10^6 cells/mL). Bright field (A) and confocal laser scanning microscopy (B to D) of the localization of 2.5 μM TMR(tetramethyl-rhodamine)-PAF26 after 30 minute peptide treatment. A to C display the same field; B is the raw unprocessed confocal image while C was digitally processed to enhance signal visualization. D shows selected cells from C, in order to illustrate the sequential mechanism of interaction, internalization and killing caused by TMR-PAF26 (1 to 5): 1, interaction with the outer cell envelopes; 2, intracellular location in small vesicles; 3, location into a big central vacuole; 4, extrusion from the vacuole to the cytosol; and 5, complete intracellular staining. (see color insert)

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

To date, one of the antifungal peptides whose internalization mechanism coupled with mode of action has been studied in more detail is the human histatin 5 against the pathogenic fungus *C. albicans*. A functional histatin 5 binding domain in SSA2p (see above) is required for peptide internalization and fungicidal activity (*59*). Furthermore, an inactive fragment of histatin 5 bound *C. albicans* CW but did not translocate into the cell, indicating that CW binding is necessary but not sufficient for cell penetration (*118*). Histatin 5 entry itself does not disrupt cell membrane integrity and precedes the entrance of the death marker propidium iodide (*83*, *119*). A role of vacuole containment in limiting peptide toxicity has been postulated, although it is controversial and difficult to establish (*83*, *119*).

Different synthetic AMP have also been demonstrated to penetrate into susceptible fungal cells and locate intracellularly, such as PAF26 in *P. digitatum* (*74*), Sub5 in *A. nidulans* (*120*) or VS2 and VS3 in *C. albicans* (*64*) (Figure 1A). All these peptides are cationic and also contain a significant number of aromatic residues (tryptophan or phenylalanine). Among them, the interaction of the hexapeptide PAF26 with *S. cerevisiae* resembles some aspects and entry routes of histatin 5 (Figure 2).

Similarly to the above mentioned CPP, antifungal peptide internalization could follow at least two pathways that probably coexist: endocytic internalization to endosomes and vacuoles or direct diffusion to the cytoplasm. However, the precise mechanisms whereby these AMP translocate across the plasma membrane remain unknown. It might vary from peptide to peptide and depend on the local peptide concentration. In some internalization studies, endocytosis has been implicated on the basis of temperature dependence, use of specific inhibitors or co-localization with endocytic probes (*115*, *119*, *121*). In the case of histatin 5, the role of endocytosis is under debate. Microscopic observation of internalized peptide in endocytosis defective mutants (*119*) and kinetic localization (*83*) suggest an endocytic pathway. However, these studies failed to reveal any significant differences in sensitivity to histatin 5 in six endocytosis-related mutants, even though some of them showed alterations in the intracellular location of the peptide. In contrast, a third independent report showed increased resistance to histatin 5 in *C. albicans* mutants lacking the genes *RVS161* and *RVS167*, which encode amphiphysins involved in scission of endocytic vesicles from the plasma membrane (*122*).

The targeted delivery of macromolecules into living cells still represents a critically limiting step in the use of chemical drugs and therapies. Peptides reviewed here with cell penetrating activity can be used as molecular shuttles, opening new perspectives in the improvement/design of AMP to carry cargoes across the cellular membrane. Recently, the BP100 peptide, originally designed as an antimicrobial peptide against plant pathogens (*123*), was used as a cell penetrating agent to transport the actin-binding Lifeact peptide into the cytosol of plant cells (*124*). Interestingly, the cell penetration capabilities of BP100 had not been explored in the original description of the peptide. The potential advantages of combining cell penetration and antimicrobial properties within selected AMP remain still unexploited. Their use should allow the design of newer and more potent drugs to fight pathogenic fungi, for instance by combining specific antifungal CPP sequences and fungicidal molecules. Thus, CPP, even those not having antimicrobial properties by themselves, are expected to be a rich source of peptidic motifs to be used in the design of novel AMP. A major goal of this kind of approaches will be to study in detail cell penetration mechanisms, including the determinants (if any) of cell specificity, to allow more specific drugs.

Intracellular Killing

Cell penetrating AMP might disrupt intracellular functions once inside the cell. Owing to their cationic nature, it is obvious that most AMP can readily bind nucleic acids (DNA and RNA) *in vitro*, which might result in a broad *in vivo* inhibition of DNA synthesis, transcription and/or mRNA translation inside cells (*61*, *74*, *114*). These effects have been confirmed in some examples of antibacterial peptides, including intracellularly acting peptides (*98*, *125*), but need to be further examined in the case of antifungal peptides. It must be also considered that in nearly all the examples analyzed nucleic acid binding by known AMP is quite unspecific, at least *in vitro*, and therefore the relevance for antimicrobial activity must be still determined.

Fungi have distinct mechanism to detect and sense AMP presence, and subsequently activate intracellular responses through signaling pathways. It must be stressed that this signaling does not necessarily rely on peptide internalization, and therefore can operate also as response to peptides that do not penetrate the fungal cell or permeate the membrane. Studies have shown microorganism responses to peptide exposure that might be related to peptide toxicity and/or part of the mechanisms to counteract peptide deleterious effect. In any case, some of the reports also indicate the existence of cell signaling components that coordinate such responses. In yeast, a transmembrane receptor-like protein is required for sensitivity to osmotin and functions upstream of RAS2p in a signaling pathway that induces apoptosis after exposure to the AMP (*62*, *126*). Different fungal protein kinase signaling cascades related to CW integrity and osmotic sensing mediate the response to distinct AMP and proteins, and mutations in the corresponding genes resulted in increased sensitivity (*67*, *86*, *127*). The increased sensitivity of fungal cells deleted in components of these signaling cascades is indicative that they are part of the microbial response to peptide exposure and damage, and not necessarily linked to the peptide antimicrobial action. The involvement of these pathways seems to be dependent on specific peptides as was nicely demonstrated in the case of two related plant defensins MsDef1 and MtDef4 (*127*).

Several independent reports show the induction of apoptosis markers in yeast (*61*, *62*, *65*, *128*) or filamentous fungi (*60*) exposed to specific AMP such as insect melittin, frog dermaseptins, fungal PAF, tobacco osmotin, or human lactoferrin. In some of these reports, mutations of pro-apoptotic genes enhance resistance to AMP, supporting that induced microbial suicide is part of the peptide killing mechanism (*60–62*). Reactive oxygen species (ROS) are among these apoptosis markers, and they also can trigger cell suicide in fungi (*129*, *130*). Further studies have increased the number of AMP whose effect is associated with an increase in endogenous ROS. This also suggests an induction of intracellular signaling

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

pathways related to ROS production. Examples include N-terminal derivative peptides of the human lactoferrin (*131*), the plant defensin RsAFP2 (*132*), or the two *de novo* designed AMP VS2 and VS3 (*64*). Interestingly, in the latter example ROS induction was coincident with internalization of fluorescently labeled peptides into *C. albicans* visualized by confocal microscopy. However, the precise role of ROS in antimicrobial action remains still controversial as exemplified by histatin 5 (*133*, *134*).

The cytosolic calcium concentration is a second messenger in eukaryotic cells that regulates numerous processes in fungi, such as spore germination, tip growth, hyphal branching, sporulation, circadian clocks and response to stress (*135*). The complex calcium-calmodulin controls the biological activity of more than 30 different proteins. Thus, since it plays a fundamental role in cell biology, agents that specifically inhibit its action should have important pharmacological impact. Recently, the involvement of intracellular Ca2+ signaling in the activity of antifungal peptides has been pointed out in different reports. It was found that the plant defensin MsDef1 blocked the mammalian L-type Ca2+ channel similarly to a virally encoded and structurally unrelated antifungal toxin KP4 from *Ustilago maydis*, whereas the structurally similar MtDef2 and RsAFP2 failed to block the same channel (*136*). A transcriptomic study suggested the contribution of calcium/calcineurin signaling in the sensitivity of *Candida* to the MUC2 12-mer peptide (*137*). In addition, studies with the *A. giganteus* AFP uncovered that the fortification of the yeast CW with chitin as response to AFP is dependent on the calcium responsive transcription factor Crz1p (*91*). Finally, the antifungal protein PAF from *P. chrysogenum* was shown to disrupt cytosolic Ca2+ homeostasis in *N. crassa* (*138*).

An elegant example of intracellular target is that of the pea defensin Psd1, which in a yeast two hybrid approach interacted with several fungal nuclear proteins, including cyclin F with which also *in vitro* binding was demonstrated (*116*). The defensin translocated to the fungal nucleus (see above) and further analysis indicated that it affects normal cell cycle progression causing conidia to undergo endoduplication. A different example of intracellular mechanism relates to the ribonuclease-like properties of plant antimicrobial PR-10 proteins. The peanut RNase AhPR-10 has been shown to locate inside hyphae and kill susceptible fungi (*139*). Interestingly, a mutant devoid of RNase activity internalizes into hyphae but does not inhibit fungal growth or disrupt membrane permeability, thus separating the cell penetration from the ribonuclease activity and, further, linking this enzymatic activity and microbicidal properties.

The use of genomic tools is expected to help in the characterization of the mechanisms of AMP action, including the effects on microorganisms, the determinants of susceptibility and the identification of novel microbial targets for AMP. Recent studies have been conducted by exposing yeasts (either *S. cerevisiae* or *C. albicans*) to AMP, and analyzing the transcriptomic response or testing the susceptibility of genome-wide mutant collections (*67–70*, *91*, *137*, *140*). Conclusions need to be critically tested for their relevance to filamentous fungi. For instance, a common report in yeast is the induction by distinct AMP of the osmotic stress response regulated by the HOG protein kinase pathway, involved in osmotic tolerance and CW maintenance (*67*, *140*). However, the

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

HOG pathway does not participate in the response of *F. graminearum* to specific defensins (*127*). The two studies that included deletion mutant screens identified genes involved in intracellular protein sorting and/or vacuole targeting (*68*, *69*). Additionally, the screening of cDNA expression libraries identified genes whose overexpression influence the sensitivity of yeast to MiAMP1 purified from *Macadamia integrifolia*, although the involvement of the corresponding gene products is unclear (*141*). Notably, gene deletion mutants of the identified genes did not confer a substantial change of susceptibility to the peptide indicating that the former mutant screens might have lost significant information.

Some of these studies have also performed direct genome-wide comparisons of the effect of different AMP on *S. cerevisiae*. Examples include a dermaseptin derivative and magainin 2, both amphipathic α-helical peptides supposed to kill microbes by membrane disruption (*69*). Conclusions indicate that these two peptide actions are more complex than cell lysis and showed common and unique effects for each peptide. In another report, *S. cerevisiae* treated with sublethal concentrations of two unrelated AMP -the cell penetrating PAF26 and the cytolitic melittin- was analyzed by transcriptome profiling (*70*). A response shared by both peptides was the induction of genes involved in reinforcement of the CW, a common effect of AMP observed in parallel studies (*137*, *140*). Global genome annotation identified genes from the arginine metabolism and urea cycle as specifically induced by PAF26, but not by melittin (*70*). Gene deletion mutants in several genes of this pathway such as *ARG1* were specifically more resistant to PAF26, had a functional CW and bound as much peptide as the parental strain, thus uncoupling peptide interaction from cell killing. *ARG1* codes for the cytosolic arginosuccinate synthetase involved in the arginine and amino groups metabolism, in which cationic AMP are rich.

Concluding Remarks

From the information discussed in this chapter, a general model for the mechanism of action of non-lytic antifungal peptides can be established. At least three different steps can be hypothesized that relate with antimicrobial activity: interaction with fungal cell, internalization and intracellular killing. However, we must be cautious as these three properties/steps may not co-exist in all the antifungal peptides. Indeed, there are examples that indicate this is not always the case. For instance, in the tobacco osmotin attraction to CW and interaction with a membrane receptor results in cell signaling that kills the fungus intracellularly (*62*, *126*), apparently in the absence of cell penetration.

Interestingly, these steps have been interrupted/disconnected in selected AMP with the use of peptide sequence variants, resulting in absence or strong reduction of antimicrobial activity. Examples include an inactive analog of histatin 5 that bound to CW but was not internalized (*118*), or the above mentioned apidaecin and PR-10 in which internalization and killing activities seem to be separated, demonstrating that peptide uptake was necessary but not sufficient for activity (*98*, *139*). An attractive hypothesis is therefore that this class of bioactive peptides are in fact modular molecules in which interaction, internalization and

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

antimicrobial determinants do not necessarily overlap each other in the peptide sequence (*66*). Additional experiments should question the existence and address the identification of these (separate) determinants on model AMP, in order to establish the minimum amino acid sequence requirements for these activities, and help in the design of "modular" domains with distinct functional capabilities. These modular domains from different AMP could be combined to make hybrid peptides with enhanced properties.

Current antifungal approaches face many difficulties both in clinic and in agriculture, including fast growing drug-resistance, limited choice of antifungal drugs and fungicides, increased fungal infections in immunosuppressed patients with AIDS, organ transplant or cancer, and safety and environmental concerns to the massive use of fungicides in crop protection. Although AMP and their analogs are promising alternatives for the control of fungal infections more effectively, further research and development are needed to bring this potential to reality. Not only a complete characterization of the mode of antifungal action, as reviewed in this chapter, is necessary, the cost effective production of AMP of desired purity is also pivotal and will depend very significantly on the final use of the peptides. The synthetic production can be affordable in the case of the clinical use, but is prohibitive for agricultural and food applications of AMP. For these latter practical uses, the promise of biotechnological production still needs to be confirmed and demonstrated feasible. With the many promising antifungal peptides that show selective toxicity toward pathogenic fungi, we anticipate that peptide analogs as well as *de novo* designed AMP will continue to be developed with increased efficacy in fighting fungal infections.

Acknowledgments

We apologize to all the colleagues whose research could not be appropriately cited due to space limitations. We thank Adokiye Berepiki (University of Edinburgh, UK) for his comments and critical review. Our work on antimicrobial peptides has been supported by grants BIO2009-12919 and EUI2008-03619 from the Spanish Ministry of Science and Innovation (MICINN), and PROMETEO2010-010 and ACOMP2011-258 from *Generalitat Valenciana*. E.H. is recipient of a fellowship from the JAE-PREDOC Program (CSIC, Spain), and A.M. of a fellowship from the Postdoctoral Mobility Program (MICINN).

References

1. Zasloff, M. *Nature* **2002**, *415*, 389–395.
2. Hancock, R. E. W.; Sahl, H. G. *Nat. Biotech.* **2006**, *24*, 1551–1557.
3. Peschel, A.; Sahl, H. G. *Nat. Rev. Microbiol.* **2006**, *4*, 529–536.
4. Marcos, J. F.; Muñoz, A.; Pérez-Payá, E.; Misra, S.; López-García, B. *Annu. Rev. Phytopathol.* **2008**, *46*, 273–301.
5. Montesinos, E. *FEMS Microbiol. Lett.* **2007**, *270*, 1–11.
6. Cotter, P. D.; Hill, C.; Ross, R. P. *Nat. Rev. Microbiol.* **2005**, *3*, 777–788.
7. Rydlo, T.; Miltz, J.; Mor, A. *J. Food Sci.* **2006**, *71*, 125–135.

8. De Lucca, A. J.; Walsh, T. J. *Antimicrob. Agents Chemother.* **1999**, *43*, 1–11.
9. Hultmark, D. *Trends Genet.* **1993**, *9*, 178–183.
10. De Lucca, A. J.; Bland, J. M.; Jacks, T. J.; Grimm, C.; Cleveland, T. E.; Walsh, T. J. *Antimicrob. Agents Chemother.* **1997**, *41*, 481–483.
11. Donlan, R. M.; Costerton, J. W. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
12. Cavallarín, L.; Andreu, D.; San Segundo, B. *Mol. Plant-Microbe Interact.* **1998**, *11*, 218–227.
13. Owens, L. D.; Heutte, T. M. *Mol. Plant-Microbe Interact.* **1997**, *10*, 525–528.
14. Yount, N. Y.; Yeaman, M. R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 7363–7368.
15. Wilmes, M.; Cammue, B. P. A.; Sahl, H. G.; Thevissen, K. *Nat. Prod. Rep.* **2011**, *28*, 1350–1358.
16. Ganz, T. *Nat. Rev. Immuno.* **2003**, *3*, 710–720.
17. Lehrer, R. I. *Nat. Rev. Microbiol.* **2004**, *2*, 727–738.
18. Schroeder, B. O.; Wu, Z.; Nuding, S.; Groscurth, S.; Marcinowski, M.; Beisner, J.; Buchner, J.; Schaller, M.; Stange, E. F.; Wehkamp, J. *Nature* **2011**, *469*, 419–423.
19. Carvalho, A.; Gomes, V. M. *Peptides* **2009**, *30*, 1007–1020.
20. Lay, F. T.; Anderson, M. A. *Curr. Protein Pept. Sci.* **2005**, *6*, 85–101.
21. Thomma, B. P. H. J.; Cammue, B. P. A.; Thevissen, K. *Planta* **2002**, *216*, 193–202.
22. Aerts, A. M.; Francois, I. E. J. A.; Cammue, B. P. A.; Thevissen, K. *Cell. Mol. Life Sci.* **2008**, *65*, 2069–2079.
23. Broekaert, W. F.; Terras, F. R. G.; Cammue, B. P. A.; Osborn, R. W. *Plant Physiol.* **1995**, *108*, 1353–1358.
24. Gao, A. G.; Hakimi, S. M.; Mittanck, C. A.; Wu, Y.; Woerner, B. M.; Stark, D. M.; Shah, D. M.; Liang, J. H.; Rommens, C. M. T. *Nat. Biotechnol.* **2000**, *18*, 1307–1310.
25. Abad, L. R.; D'Urzo, M. P.; Liu, D.; Narasimhan, M. L.; Reuveni, M.; Zhu, J. K.; Niu, X.; Singh, N. K.; Hasegawa, P. M.; Bressan, R. A. *Plant Sci.* **1996**, *118*, 11–23.
26. Mygind, P. H.; Fischer, R. L.; Schnorr, K. M.; Hansen, M. T.; Sonksen, C. P.; Ludvigsen, S.; Raventos, D.; Buskov, S.; Christensen, B.; De Maria, L.; Taboureau, O.; Yaver, D.; Elvig-Jorgensen, S. G.; Sorensen, M. V.; Christensen, B. E.; Kjaerulff, S.; Frimodt-Moller, N.; Lehrer, R. I.; Zasloff, M.; Kristensen, H. H. *Nature* **2005**, *437*, 975–980.
27. Zhu, S. *Mol. Immunol.* **2008**, *45*, 828–838.
28. Lacadena, J.; Martínez del Pozo, A.; Gasset, M.; Patino, B.; Campos-Olivas, R.; Vázquez, C.; Martínez-Ruiz, A.; Mancheño, J. M.; Oñaderra, M.; Gavilanes, J. G. *Arch. Biochem. Biophys.* **1995**, *324*, 273–281.
29. Nakaya, K.; Omata, K.; Okahashi, I.; Nakamura, Y.; Kolekenbrock, H.; Ulbrich, N. *Eur. J. Biochem.* **1990**, *193*, 31–38.
30. Vila, L.; Lacadena, V.; Fontanet, P.; Martínez del Pozo, A.; San Segundo, B. *Mol. Plant-Microbe Interact.* **2001**, *14*, 1327–1331.
31. Meyer, V. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 17–28.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

32. Marx, F.; Haas, H.; Reindl, M.; Stoffler, G.; Lottspeich, F.; Redl, B. *Gene* **1995**, *167*, 167–171.
33. Kaiserer, L.; Oberparleiter, C.; Weiler-Gorz, R.; Burgstaller, W.; Leiter, E.; Marx, F. *Arch. Microbiol.* **2003**, *180*, 204–210.
34. Gun Lee, D.; Yub Shin, S.; Maeng, C. Y.; Zhu Jin, Z.; Lyong Kim, K.; Hahm, K. S. *Biochem. Biophys. Res. Commun.* **1999**, *263*, 646–651.
35. Geisen, R. *Int. J. Food Microbiol.* **2000**, *62*, 95–101.
36. Kovács, L.; Virágh, M.; Takó, M.; Papp, T.; Vágvölgyi, C.; Galgóczy, L. *Peptides* **2011**, *32*, 1724–1731.
37. Skouri-Gargouri, H.; Gargouri, A. *Peptides* **2008**, *29*, 1871–1877.
38. Marx, F.; Binder, U.; Leiter, É.; Pócsi, I. *Cell. Mol. Life Sci.* **2008**, *65*, 445–454.
39. López-García, B.; Moreno, A. B.; San Segundo, B.; De los Ríos, V.; Manning, J. M.; Gavilanes, J. G.; Martínez del Pozo, A. *Protein Expression Purif.* **2010**, *70*, 206–210.
40. Batta, G.; Barna, T.; Gáspári, Z.; Sándor, S.; Kövér, K. E.; Binder, U.; Sarg, B.; Kaiserer, L.; Chhillar, A. K.; Eigentler, A.; Leiter, E.; Hegedüs, N.; Pócsi, I.; Lindner, H.; Marx, F. *FEBS J.* **2009**, *276*, 2875–2890.
41. Oppenheim, F. G.; Xu, T.; Mcmillian, F. M.; Levitz, S. M.; Diamond, R. D.; Offner, G. D.; Troxler, R. F. *J. Biol. Chem.* **1988**, *263*, 7472–7477.
42. Xu, T.; Levitz, S. M.; Diamond, R. D.; Oppenheim, F. G. *Infect. Immun.* **1991**, *59*, 2549–2554.
43. Helmerhorst, E. J.; Reijnders, I. M.; Van't Hof, W.; Simoons-Smit, I.; Veerman, E. C. I.; Amerongen, A. V. N. *Antimicrob. Agents Chemother.* **1999**, *43*, 702–704.
44. Rothstein, D. M.; Spacciapoli, P.; Tran, L. T.; Xu, T.; Roberts, F. D.; Dalla Serra, M.; Buxton, D. K.; Oppenheim, F. G.; Friden, P. *Antimicrob. Agents Chemother.* **2001**, *45*, 1367–1373.
45. De Lucca, A. J.; Bland, J. M.; Grimm, C.; Jacks, T. J.; Cary, J. W.; Jaynes, J. M.; Cleveland, T. E.; Walsh, T. J. *Can. J. Bot.* **1998**, *44*, 514–520.
46. López-García, B.; Pérez-Payá, E.; Marcos, J. F. *Appl. Environ. Microbiol.* **2002**, *68*, 2453–2460.
47. Walker, L. A.; Gow, N. A. R.; Munro, C. A. *Fungal Genet. Biol.* **2010**, *47*, 117–126.
48. Denning, D. W. *J. Antimicrob. Chemother.* **2002**, *49*, 889–891.
49. Benz, F.; Knusel, F.; Nuesch, J.; Treichler, H. J.; Voser, W. *Helv. Chim. Acta* **1974**, *57*, 2459–2477.
50. Brogden, K. A. *Nat. Rev. Microbiol.* **2005**, *3*, 238–250.
51. Hale, J. D.; Hancock, R. E. W. *Expert Rev. Anti-Infect. Ther.* **2007**, *5*, 951–959.
52. Jenssen, H.; Hamill, P.; Hancock, R. E. W. *Clin. Microbiol. Rev.* **2006**, *19*, 491–511.
53. Marcos, J. F.; Gandía, M. *Expert Opin. Drug Discovery* **2009**, *4*, 659–671.
54. Yount, N. Y.; Bayer, A. S.; Xiong, Y. Q.; Yeaman, M. R. *Biopolymers* **2006**, *84*, 435–458.
55. Schneider, T.; Kruse, T.; Wimmer, R.; Wiedemann, I.; Sass, V.; Pag, U.; Jansen, A.; Nielsen, A. K.; Mygind, P. H.; Raventos, D. S.;

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Neve, S.; Ravn, B.; Bonvin, A. M. J. J.; De Maria, L.; Andersen, A. S.; Gammelgaard, L. K.; Sahl, H. G.; Kristensen, H. H. *Science* **2010**, *328*, 1168–1172.

56. Breukink, E.; Wiedemann, I.; van Kraaij, C.; Kuipers, O. P.; Sahl, H. G.; de Kruijff, B. *Science* **1999**, *286*, 2361–2364.
57. Thevissen, K.; Warnecke, D. C.; François, I. E. J. A.; Leipelt, M.; Heinz, E.; Ott, C.; Zahringer, U.; Thomma, B. P. H. J.; Ferkel, K. K. A.; Cammue, B. P. A. *J. Biol. Chem.* **2004**, *279*, 3900–3905.
58. Kragol, G.; Lovas, S.; Varadi, G.; Condie, B. A.; Hoffmann, R.; Otvos, L., Jr. *Biochemistry* **2001**, *40*, 3016–3026.
59. Sun, J. N. N.; Li, W. S.; Jang, W. S.; Nayyar, N.; Sutton, M. D.; Edgerton, M. *Mol. Microbiol.* **2008**, *70*, 1246–1260.
60. Leiter, E.; Szappanos, H.; Oberparleiter, C.; Kaiserer, L.; Csernoch, L.; Pusztahelyi, T.; Emri, T.; Posci, I.; Salvenmoser, W.; Marx, F. *Antimicrob. Agents Chemother.* **2005**, *49*, 2445–2453.
61. Morton, C. O.; dos Santos, S. C.; Coote, P. *Mol. Microbiol.* **2007**, *65*, 494–507.
62. Narasimhan, M. L.; Damsz, B.; Coca, M. A.; Ibeas, J. I.; Yun, D. J.; Pardo, J. M.; Hasegawa, P. M.; Bressan, R. A. *Mol. Cell* **2001**, *8*, 921–930.
63. Mello, E. O.; Ribeiro, S. F. F.; Carvalho, A. O.; Santos, I. S.; Da Cunha, M.; Santa-Catarina, C.; Gomes, V. M. *Curr. Microbiol.* **2011**, *62*, 1209–1217.
64. Maurya, I. K.; Pathak, S.; Sharma, M.; Sanwal, H.; Chaudhary, P.; Tupe, S.; Deshpande, M.; Chauhan, V. S.; Prasad, R. *Peptides* **2011**, *32*, 1732–1740.
65. Park, C.; Lee, D. G. *Biochem. Biophys. Res. Commun.* **2010**, *394*, 170–172.
66. Otvos, L., Jr. *J. Pept. Sci.* **2005**, *11*, 697–706.
67. Vylkova, S.; Jang, W. S.; Li, W. S.; Nayyar, N.; Edgerton, M. *Eukaryot. Cell* **2007**, *6*, 1876–1888.
68. Lis, M.; Fuss, J. R.; Bobek, L. A. *Antimicrob. Agents Chemother.* **2009**, *53*, 3762–3769.
69. Morton, C. O.; Hayes, A.; Wilson, M.; Rash, B. M.; Oliver, S. G.; Coote, P. *Antimicrob. Agents Chemother.* **2007**, *51*, 3948–3959.
70. López-García, B.; Gandía, M.; Muñoz, A.; Carmona, L.; Marcos, J. F. *BMC Microbiol.* **2010**, *10*, 289.
71. Bowman, S. M.; Free, S. J. *Bioessays* **2006**, *28*, 799–808.
72. Debono, M.; Gordee, R. S. *Annu. Rev. Microbiol.* **1994**, *48*, 471–497.
73. Odds, F. C.; Brown, A. J. P.; Gow, N. A. R. *Trends Microbiol.* **2003**, *11*, 272–279.
74. Muñoz, A.; López-García, B.; Marcos, J. F. *Antimicrob. Agents Chemother.* **2006**, *50*, 3847–3855.
75. Liu, X.; Wang, J.; Gou, P.; Mao, C.; Zhu, Z. R.; Li, H. *Int. J. Food Microbiol.* **2007**, *119*, 223–229.
76. Oard, S.; Rush, M. C.; Oard, J. H. *J. Appl. Microbiol.* **2004**, *97*, 169–180.
77. Ali, G. S.; Reddy, A. S. *Mol. Plant-Microbe Interact.* **2000**, *13*, 847–859.
78. Sagaram, U. S.; Pandurangi, R.; Kaur, J.; Smith, T. J.; Shah, D. M. *PLoS One* **2011**, *6*.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

79. Yun, D. J.; Zhao, Y.; Pardo, J. M.; Narasimhan, M. L.; Damsz, B.; Lee, H.; Abad, L. R.; Durzo, M. P.; Hasegawa, P. M.; Bressan, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 7082–7087.
80. Dielbandhoesing, S. K.; Zhang, H.; Caro, L. H.; van der Vaart, J. M.; Klis, F. M.; Verrips, C. T.; Brul, S. *Appl. Environ. Microbiol.* **1998**, *64*, 4047–4052.
81. van der Weerden, N. L.; Hancock, R. E. W.; Anderson, M. A. *J. Biol. Chem.* **2010**, *285*, 37513–37520.
82. Li, X. S.; Reddy, M. S.; Baev, D.; Edgerton, M. *J. Biol. Chem.* **2003**, *278*, 28553–28561.
83. Jang, W. S.; Bajwa, J. S.; Sun, J. N.; Edgerton, M. *Mol. Microbiol.* **2010**, *77*, 354–370.
84. Vylkova, S.; Li, X. S.; Berner, J. C.; Edgerton, M. *Antimicrob. Agents Chemother.* **2006**, *50*, 324–331.
85. Ibeas, J. I.; Lee, H.; Damsz, B.; Prasad, D. T.; Pardo, J. M.; Hasegawa, P. M.; Bressan, R. A.; Narasimhan, M. L. *Plant J.* **2000**, *23*, 375–383.
86. Koo, J. C.; Lee, B.; Young, M. E.; Koo, S. C.; Cooper, J. A.; Baek, D.; Lim, C. O.; Lee, S. Y.; Yun, D. J.; Cho, M. J. *Plant Cell Physiol.* **2004**, *45*, 1669–1680.
87. Harris, M.; Mora-Montes, H. M.; Gow, N. A. R.; Coote, P. J. *Microbiology (UK)* **2009**, *155*, 1058–1070.
88. Fujimura, M.; Ideguchi, M.; Minami, Y.; Watanabe, K.; Tadera, K. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 642–645.
89. Hagen, S.; Marx, F.; Ram, A. F.; Meyer, V. *Appl. Environ. Microbiol.* **2007**, *73*, 2128–2134.
90. Martín-Urdiroz, M.; Martínez-Rocha, A. L.; Di Pietro, A.; Martínez del Pozo, A.; Roncero, M. I. G. *Int. Microbiol.* **2009**, *12*, 115–121.
91. Ouedraogo, J. P.; Hagen, S.; Spielvogel, A.; Engelhardt, S.; Meyer, V. *J. Biol. Chem.* **2011**, *286*, 13859–13868.
92. Thevissen, K.; Idkowiak-Baldys, J.; Im, Y. J.; Takemoto, J.; François, I. E. J. A.; Ferket, K. K. A.; Aerts, A. M.; Meert, E. M. K.; Winderickx, J.; Roosen, J.; Cammue, B. P. A. *FEBS Lett.* **2005**, *579*, 1973–1977.
93. Thevissen, K.; Cammue, B. P. A.; Lemaire, K.; Winderickx, J.; Dickson, R. C.; Lester, R. L.; Ferket, K. K.; Van Even, F.; Parret, A. H.; Broekaert, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9531–9536.
94. Stock, S. D.; Hama, H.; Radding, J. A.; Young, D. A.; Takemoto, J. Y. *Antimicrob. Agents Chemother.* **2000**, *44*, 1174–1180.
95. Aerts, A. M.; François, I. E. J. A.; Bammens, L.; Cammue, B. P. A.; Smets, B.; Winderickx, J.; Accardo, S.; De Vos, D. E.; Thevissen, K. *FEBS Lett.* **2006**, *580*, 1903–1907.
96. Thevissen, K.; Yen, W. L.; Carmona-Gutierrez, D.; Idkowiak-Baldys, J.; Aerts, A. M.; François, I. E. J. A.; Madeo, F.; Klionsky, D. J.; Hannun, Y. A.; Cammue, B. P. A. *FEMS Microbiol. Lett.* **2010**, *303*, 164–168.
97. Ramamoorthy, V.; Cahoon, E. B.; Jia, L.; Thokala, M.; Minto, R. E.; Shah, D. M. *Mol. Microbiol.* **2007**, *66*, 771–786.
98. Castle, M.; Nazarian, A.; Yi, S. S.; Tempst, P. *J. Biol. Chem.* **1999**, *274*, 32555–32564.

99. Park, C. B.; Yi, K. S.; Matsuzaki, K.; Kim, M. S.; Kim, S. C. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8245–8250.
100. Scocchi, M.; Tossi, A.; Gennaro, R. *Cell. Mol. Life Sci.* **2011**, *68*, 2317–2330.
101. Lindgren, M. E. *Trends Pharmacol. Sci.* **2000**, *21*, 99–103.
102. Jarver, P.; Langel, U. *Biochim. Biophys. Acta, Biomembr.* **2006**, *1758*, 260–263.
103. Jung, H. J.; Park, Y.; Hahm, K. S.; Lee, D. G. *Biochem. Biophys. Res. Commun.* **2006**, *345*, 222–228.
104. Henriques, S. T.; Melo, M. N.; Castanho, M. A. R. B. *Biochem. J.* **2006**, *399*, 1–7.
105. Herce, H. D.; Garcia, A. E. *J. Biol. Phys.* **2007**, *33*, 345–356.
106. Nicolas, P. *FEBS J.* **2009**, *276*, 6483–6496.
107. Splith, K.; Neundorf, I. *Eur. Biophys. J.* **2011**, *40*, 387–397.
108. Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. *J. Biol. Chem.* **2003**, *278*, 585–590.
109. Alves, I. D.; Jiao, C. Y.; Aubry, S.; Aussedat, B.; Burlina, F.; Chassaing, G.; Sagan, S. *Biochim. Biophys. Acta, Biomembr.* **2010**, *1798*, 2231–2239.
110. Fischer, R.; Fotin-Mleczek, M.; Hufnagel, H.; Brock, R. *ChemBioChem* **2005**, *6*, 2126–2142.
111. Duchardt, F.; Fotin-Mleczek, M.; Schwarz, H.; Fischer, R.; Brock, R. *Traffic* **2007**, *8*, 848–866.
112. Gould, G. W.; Lippincott-Schwartz, J. *Nat. Rev. Molec. Cell Biol.* **2009**, *10*, 287–292.
113. Verdurmen, W. P. R.; Brock, R. *Trends Pharmacol. Sci.* **2011**, *32*, 116–124.
114. Moreno, A. B.; Martínez del Pozo, A.; San Segundo, B. *App. Microbiol. Biotechnol.* **2006**, *72*, 883–895.
115. Oberparleiter, C.; Kaiserer, L.; Haas, H.; Ladurner, P.; Andratsch, M.; Marx, F. *Antimicrob. Agents Chemother.* **2003**, *47*, 3598–3601.
116. Lobo, D. S.; Pereira, I. B.; Fragel-Madeira, L.; Medeiros, L. N.; Cabral, L. M.; Faria, J.; Bellio, M.; Campos, R. C.; Linden, R.; Kurtenbach, E. *Biochemistry* **2007**, *46*, 987–996.
117. van der Weerden, N. L.; Lay, F. T.; Anderson, M. A. *J. Biol. Chem.* **2008**, *283*, 14445–14452.
118. Jang, W. S.; Li, X. W. S.; Sun, J. N. N.; Edgerton, M. *Antimicrob. Agents Chemother.* **2008**, *52*, 497–504.
119. Mochon, A. B.; Liu, H. P. *PLoS Pathog.* **2008**, *4*, e1000190.
120. Mania, D.; Hilpert, K.; Ruden, S.; Fischer, R.; Takeshita, N. *Appl. Environ. Microbiol.* **2010**, *76*, 7102–7108.
121. Zhu, J.; Luther, P. W.; Leng, Q.; Mixson, A. J. *Antimicrob. Agents Chemother.* **2006**, *50*, 2797–2805.
122. Douglas, L. M.; Martin, S. W.; Konopka, J. B. *Infect. Immun.* **2009**, *77*, 4150–4160.
123. Ferre, R.; Badosa, E.; Feliu, L.; Planas, M.; Montesinos, E.; Bardají, E. *Appl. Environ. Microbiol.* **2006**, *72*, 3302–3308.
124. Eggenberger, K.; Mink, C.; Wadhwani, P.; Ulrich, A. S.; Nick, P. *ChemBioChem* **2011**, *12*, 132–137.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

125. Park, C. B.; Kim, H. S.; Kim, S. C. *Biochem. Biophys. Res. Commun.* **1998**, *244*, 253–257.
126. Narasimhan, M. L.; Coca, M. A.; Jin, J. B.; Yamauchi, T.; Ito, Y.; Kadowaki, T.; Kim, K. K.; Pardo, J. M.; Damsz, B.; Hasegawa, P. M.; Yun, D. J.; Bressan, R. A. *Mol. Cell* **2005**, *17*, 171–180.
127. Ramamoorthy, V.; Zhao, X. H.; Snyder, A. K.; Xu, J. R.; Shah, D. M. *Cell. Microbiol.* **2007**, *9*, 1491–1506.
128. Andrés, M. T.; Viejo-Díaz, M.; Fierro, J. F. *Antimicrob. Agents Chemother.* **2008**, *52*, 4081–4088.
129. Madeo, F.; Herker, E.; Wissing, S.; Jungwirth, H.; Eisenberg, T.; Frohlich, K. U. *Curr. Opin. Microbiol.* **2004**, *7*, 655–660.
130. Perrone, G. G.; Tan, S. X.; Dawes, I. W. *Biochim. Biophys. Acta, Mol. Cell Res.* **2008**, *1783*, 1354–1368.
131. Lupetti, A.; Paulusma-Annema, A.; Senesi, S.; Campa, M.; van Dissel, J. T.; Nibbering, P. H. *Antimicrob. Agents Chemother.* **2002**, *46*, 1634–1639.
132. Aerts, A. M.; François, I. E. J. A.; Meert, E. M. K.; Li, Q. T.; Cammue, B. P. A.; Thevissen, K. *J. Mol. Microbiol. Biotechnol.* **2007**, *13*, 243–247.
133. Helmerhorst, E. J.; Troxler, R. F.; Oppenheim, F. G. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14637–14642.
134. Veerman, E. C. I.; Nazmi, K.; van Hof, W.; Bolscher, J. G. M.; den Hertog, A. L.; Amerongen, A. V. N. *Biochem. J.* **2004**, *381*, 447–452.
135. Miller, A. J.; Vogg, G.; Sanders, D. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9348–9352.
136. Spelbrink, R. G.; Dilmac, N.; Allen, A.; Smith, T. J.; Shah, D. M.; Hockerman, G. H. *Plant Physiol.* **2004**, *135*, 2055–2067.
137. Lis, M.; Liu, T. T.; Barker, K. S.; Rogers, P. D.; Bobek, L. A. *FEMS Yeast Res.* **2010**, *10*, 579–586.
138. Binder, U.; Chu, M.; Read, N. D.; Marx, F. *Eukaryot. Cell* **2010**, *9*, 1374–1382.
139. Chadha, P.; Das, R. H. *Planta* **2006**, *225*, 213–222.
140. Gamberi, T.; Cavalieri, D.; Magherini, F.; Mangoni, L.; De Filippo, C.; Borro, M.; Gentile, G.; Simmaco, M.; Modesti, A. *Biochim. Biophys. Acta, Proteins Proteomics* **2007**, *1774*, 688–700.
141. Stephens, C.; Harrison, S. J.; Kazan, K.; Smith, F. W. N.; Goulter, K. C.; Maclean, D. J.; Manners, J. M. *Curr. Genet.* **2005**, *47*, 194–201.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch016

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

Chapter 17

Thionin Antifungal Peptide Synthesis in Transgenic Barley

**Jianming Fu,[1,§] Minesh Patel,[2] Anna Maria Nuutila,[3]
and Ron Skadsen*,[4,§]**

**[1]Current address: USDA-ARS Hard Winter Wheat Genetics
Research Unit, 4008 Throckmorton Hall, Kansas State University,
Manhattan, KS 66506, U.S.A.
[2]Current address: Dept. of Crop Science, 840 Main Campus Dr.,
Campus Box 7287, North Carolina State University,
Raleigh, NC 27513, U.S.A.
[3]Current address: VTT Biotechnology and Research, P.O. Box 1501
(Tietotie 2) FIN-02044 VTT, Finland
[4]Address: USDA-ARS Cereal Crops Research Unit, 502 Walnut St.,
Madison, WI 53726, U.S.A.
*E-mail: rskadsen@wisc.edu
§First authorship is shared.**

Seeds and vegetative organs of barley and other cereals produce thionin proteins that are processed into peptides with pronounced anti-microbial properties. *In vitro* studies demonstrated the toxicity of α- and β-hordothionins (HTHs) to the fungal pathogen *Fusarium graminearum*. Increasing the expression of thionin genes may, therefore, provide resistance against this and other pathogens. Before a transgenic strategy can succeed, obstacles to *Hth* expression must be overcome. Barley transformed with a seed-specific barley *αHth1* gene produced very little *Hth1* mRNA in non-endosperm tissues. Removal of the first of two nearby 5′ methionine codons (producing *αHth1Δ*) increased Hth1 mRNA levels. However, not even the association of *Hth1* with membrane-bound polyribosomes led to HTH protein accumulation. Transformation of oat with this *Hth1* vector previously showed that HTH1 protein was produced, but only in the endosperm. These findings suggest that cereals

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

have a mechanism (or deficiency) that prevents HTH1 protein accumulation beyond the endosperm. A β-hordothionin cDNA, *Lemthio1*, cloned from lemma (hull) mRNA, produced high levels of LEMTHIO1 protein in lemmas of transformants. These will be tested for resistance to *F. graminearum*.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

Introduction

Cereal Thionins

Seed-specific thionins of monocots are cysteine- and lysine-rich storage proteins. In barley, they constitute up to 4% of endosperm protein (*1*). Indeed, the promoter of the endosperm-specific thionin gene *Hth-1* has extensive homologies with the B-hordein storage protein promoter (*2*). HTHs are divided into two main groups, α/β and γ-thionins, according to amino acid sequence and 3-dimensional structure (reviewed by (*3*)). However, the γ-thionins are more closely related to defensins. Since these may not be thionins (*3*, *4*), they will not be discussed here. The α/β-thionins can be divided into 5 types (I through V). Types I and II occur in barley and other cereal grains. In both, the approximately 17 kDa precursor protein consists of three domains: an N-terminal signal peptide of 24 (type I) to 28 (type II) amino acids, a 45 (type I) to 47 (type II) amino acid mature peptide, and a 64 amino acid C-terminal acidic peptide. Type I mature peptides are highly basic (pI 10.5), relative to type II (pI ca. 8.5). This allows the acidic peptide to balance the charge of the mature peptide. Both types are processed at the same sites, producing the same three domains (*5*). The signal peptide may be co-translationally cleaved (*6*), while the acidic peptide appears to be cleaved post-translationally (*7*). The mature peptides are very stable, owing to 8 cysteines that form 4 disulfide bridges. This helps to maintain a 3-dimensional shape that resembles a capital “L” (*4*). The mature peptides (referred to as HTHs, below) have pronounced antimicrobial activity.

Type I αHTHs are produced in the endosperm of developing barley seeds (reviewed in (*8*)). They are found externally associated with protein bodies (*9*), perhaps as part of the protein-lipid matrix in which they are embedded (*10*), or externally associated with the ER (*6*). Type II thionins are found in vegetative organs, such as leaves, roots and lemmas. They are associated with the leaf cell walls or the vacuoles – the latter being 40-fold more prevalent than the former (*6*, *11*). Barley *αHth* genes are present at 2-4 copies per haploid genome, while type II hordothionin genes are variously estimated from 9-11 gene copies (*12*) or up to 50-100 copies (*5*).

Toxicity of HTH to *F. graminearum* and Other Pathogens

Barley HTH and other thionins are toxic to a wide variety of fungal and bacterial pathogens (*8*). As such, there have been attempts to produce resistance through genetic transformation. αHTH has been produced in transgenic tobacco, enhancing resistance to the pathogenic fungus *Pseudomonas syringae* (*13*, *14*). A barley β-hordothionin (type II) gene was used to transform tobacco, producing resistance to *Botrytis cinerea* and *Pseudomonas solanacearum* (*15*). The induction of type II *Hths* by fungi, and their antifungal properties *in vitro* (*16*), also suggest that these peptides can be used to produce disease resistance. The Arabidopsis *Thi2.1* thionin gene was found to be inducible by pathogenic fungi, and its constitutive over-expression led to resistance to *F. oxysporum* (*14*). *In vitro* assays have also been used to determine the antifungal potential of HTH peptides. Both the vacuolar and cell wall forms of barley type II thionin were lethal to the sugar cane fungal pathogen, *Thielaviopsis paradoxica* (*11*). However, the lethal concentration was impractically high - 2.5 mg per ml. A hordothionin gene (*Hth*) was expressed in tobacco, and the HTH purified from these plants had *in vitro* activity against *Clavibacter michiganensis* (*16*). The precise mechanism of thionin toxicity is unknown. It is known to disrupt membranes and probably causes ion channel formation (*17*).

The rationale for over-expressing thionins in transgenic barley is that *F. graminearum* infects developing seed spikelets mainly through the exposed seed tip and also through the lemma and palea which develop into the hull (*18*). HTH1 is located in the starchy endosperm where it cannot play a role in preventing infection. We have found that HTH is highly toxic to *F. graminearum* at concentrations achievable *in planta*. Since HTH is safely consumed in most diets, it is a good peptide candidate for strategies to produce pathogen resistance in field conditions. Before any strategy can succeed, it is necessary to understand the requirements for expressing thionins in target tissues.

Results

Toxicity of HTH

Prior to undertaking cloning and genetic transformation, it was necessary to determine whether HTH was indeed toxic to *F. graminearum*. Assays were conducted in which large well microtitre plates were inoculated with conidiospores and increasing amounts of various HTH preparations. Purified αHTH from barley endosperm (provided by Berne Jones, ARS) clearly disrupted growth at only 5.2 µg/mL and was 100% lethal between 5.2 and 16 µg/mL (Figure 1A). A βHTH peptide, LEMTHIO1, was also tested (Figure 1B; see below). Due to its extremely low concentrations in lemmas, the peptide was synthesized and purified by HPLC (U. of Wisconsin Biotechnology Center). This was 100% lethal between 80 and 160 µg/mL. In contrast, synthetic αHTH was not as effective, being 100% lethal between 160 and 320 µg/mL. The HPLC elution patterns following peptide synthesis indicated that these differences were probably due to the fidelity of disulfide bridge formation. LEMTHIO1 eluted as one sharp peak, while synthesized αHTH1 eluted in a long broad pattern (data not shown). Therefore, LEMTHIO1 probably assumed the correct cys-cys bons. αHTH apparently formed bridges at random, leaving only one correctly folded peptide fraction. This may have accounted for all of the toxicity in the preparation. This also indicated that αHTH has more stringent requirements for folding. Also, HTH1 may not have folded correctly, simply due to the C to N direction of chemical synthesis, whereas LEMTHIO1 may have folded correctly

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

when synthesized from either direction. To estimate the potential lethality of αHTH1 *in vivo*, a small scale purification was conducted with 20 seeds. The αHTH1 concentration required for 100% lethality was the equivalent of that found in 1 to 4 seed endosperms (Figure 1C). We also found that *F. graminearum* hyphae did not cross from the epicarp into the endosperm for at least two weeks during spikelet infections in intact plants (*18*). Therefore, if roughly 10 µg per mL of HTH1 can be produced in the epicarp and lemma, Fusarium growth should be sufficiently inhibited to prevent production of harmful levels of mycotoxins.

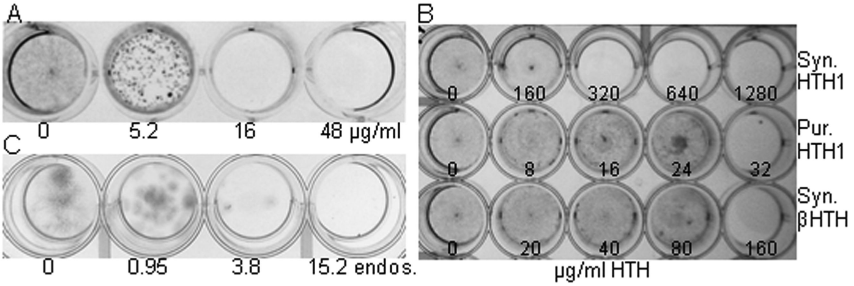
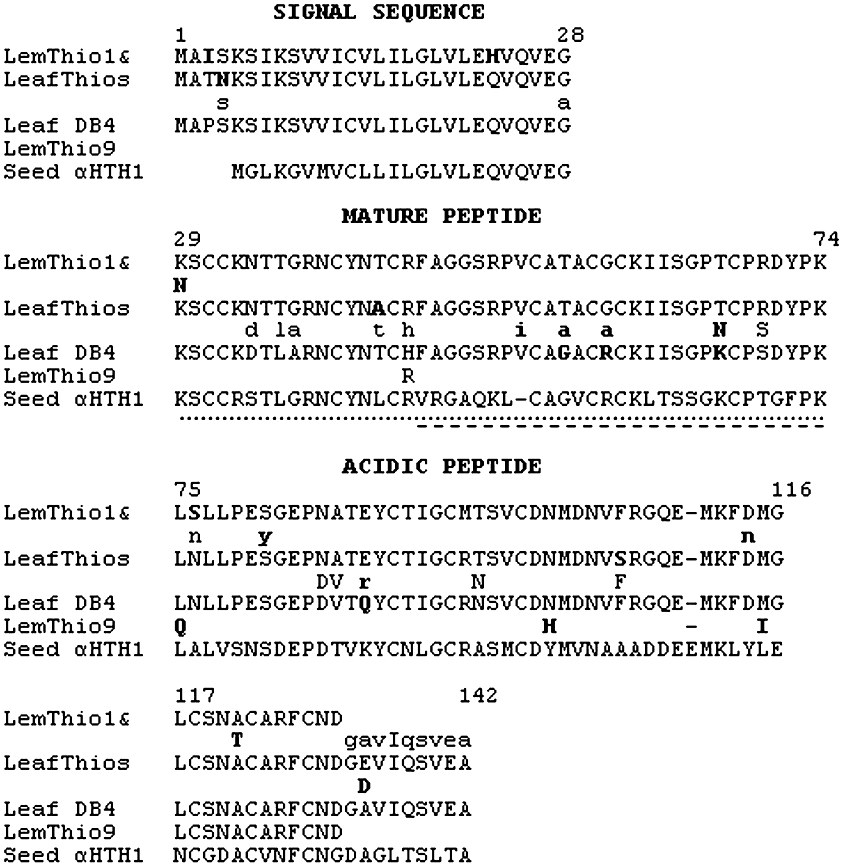


Figure 1. Toxicity of hordothionins against F. graminearum after two days of incubation. ***Panel A:*** *HTH1 mature peptide tested at various concentrations against 4000 F. graminearum conidiospores per well. HTH1 was purified from seed and was 100% lethal at a concentration between 5.2 and 16 µg/ml.* ***Panel B:*** *Toxicity of synthetic HTH1 (top row), purified seed HTH1 (middle), and synthetic LEMTHIO1 (βHTH). 4000 spores per well. Purified HTH1 was still 100% lethal between 24 and 32 µg per ml after storage in water at -18°C for one year. Synthetic LEMTHIO1 was roughly one-fourth as toxic as stored purified HTH1.* ***Panel C:*** *Determination of lethality on a per seed basis. 1000 spores per well. Approximately four developing seeds would be 100% lethal.*

Cloning of Hth cDNAs

The longest *Hth1* clone obtained from our developing seed cDNA library (Morex cv.) contained a 29 bp 5′ UTR (untranslated region), an open reading frame of 399 bp and a 3′ UTR of 161 bp. The coding sequence began with 2 methionine codons separated from each other by 15 b, as previously reported by others (*2*) (Figure 2). The *Hth1* transcript sequence was essentially identical to that deduced from genomic clone *Hth-1*, after removing introns (GenBank accession M23080; (*2*)). The only differences from *Hth-1* were 3 silent substitutions in the coding region, which were attributed to cultivar differences. Since the clone represented an α-hordothionin transcript, it was named *Hth1*.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017



```
                      SIGNAL SEQUENCE
               1                          28
LemThio1&      MAISKSIKSVVICVLILGLVLEHVQVEG
LeafThios      MATNKSIKSVVICVLILGLVLEQVQVEG
                  s                       a
Leaf DB4       MAPSKSIKSVVICVLILGLVLEQVQVEG
LemThio9
Seed αHTH1         MGLKGVMVCLLILGLVLEQVQVEG

                           MATURE PEPTIDE
               29                                           74
LemThio1&      KSCCKNTTGRNCYNTCRFAGGSRPVCATACGCKIISGPTCPRDYPK
               N
LeafThios      KSCCKNTTGRNCYNACRFAGGSRPVCATACGCKIISGPTCPRDYPK
                    d la     t h       i  a  a       N  S
Leaf DB4       KSCCKDTLARNCYNTCHFAGGSRPVCAGACRCKIISGPKCPSDYPK
LemThio9                       R
Seed αHTH1     KSCCRSTLGRNCYNLCRVRGAQKL-CAGVCRCKLTSSGKCPTGFPK
               ..............................................
                                -----------------------------

                           ACIDIC PEPTIDE
               75                                        116
LemThio1&      LSLLPESGEPNATEYCTIGCMTSVCDNMDNVFRGQE-MKFDMG
                n    y                                 n
LeafThios      LNLLPESGEPNATEYCTIGCRTSVCDNMDNVSRGQE-MKFDMG
                         DV r       N         F
Leaf DB4       LNLLPESGEPDVTQYCTIGCRNSVCDNMDNVFRGQE-MKFDMG
LemThio9       Q                         H         -    I
Seed αHTH1     LALVSNSDEPDTVKYCNLGCRASMCDYMVNAAADDEEMKLYLE

               117                 142
LemThio1&      LCSNACARFCND
                   T       gavIqsvea
LeafThios      LCSNACARFCNDGEVIQSVEA
                            D
Leaf DB4       LCSNACARFCNDGAVIQSVEA
LemThio9       LCSNACARFCND
Seed αHTH1     NCGDACVNFCNGDAGLTSLTA
```

Figure 2. Deduced amino acid sequences of type II βHths cloned from barley lemmas and compared with known leaf and seed sequences. The typical HTH domains are arranged in tiers (signal sequence, mature peptide, acidic peptide). Dotted and dashed lines represent sequences used to synthesize peptides for βHTH and αHTH1,respectively, for producing antibodies. LemThio1& represents LemThio18, plus 6 identical clone sequences; amino acids below this represent alternative amino acids occurring at specific positions in minority clones (the lower case sequence at 134 is derived from clone Lemthio41, which overlaps with most of LemThio18). Unique leaf type II βHTH sequences (DB4, or accession X05576), lemma type II (Lemthio9) and seed type I αHTH1 are shown for comparison. LeafThios represent the consensus sequence for the majority of 12 leaf thionin GenBank accessions; the line below represents amino acids that occur in three or fewer clones (lower case) or in at least 4 clones (upper case). Amino acids in bold are unique to lemmas or leaves - not considering seeds. Base numbers are according to the LemThio1 sequence. The sequences of cell wall (accession A33708; xxxxkqvlarlxpnixxfaggskpvxaaaxxxvii) and soluble (accession B33708; ksxxknvlgrnxyntxxpa) HTHs are not displayed but occur between bases 29 and 63.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

βHth cDNAs from lemmas were cloned using a PCR approach. This was fortuitous, as the levels of *βHth* transcripts were very low and not detectable on northern blots at normal exposures. Deduced amino acid sequences were highly conserved between lemmas and a consensus of 12 barley leaf thionin GenBank accessions ("LeafThios", Figure 2). Homology to endosperm αHTH1 was not as strong. The βHTH proteins fell into two main classes. Most lemma sequences were represented by "LemThio1&", while two were represented by LemThio9. Similarly for leaves, most sequences were represented by "LeafThios", whereas Leaf DB4 has unique amino acids at various positions. All *βHth* 5′ coding sequences began with MAT or MAP. However, we found that most lemma N-terminal sequences began with MAI. The most common transcript was named "LemThio1" (gb accession FJ026806.1). Recently, a barley leaf transcript was reported to have a MAI leader (gb accession BAJ90360.1). Barley *βHths* cloned from lemmas probably represent both vacuolar HTH and cell wall forms. It appears that the criteria used to distinguish these forms relies on sequencing of thionin peptide fragments from cell walls and purified from soluble extracts (see Figure 2 legend). These were too short and ambiguous to serve as signature sequences for either form. Based on retention in the insoluble fraction, LEMTHIO1 was classified as a cell wall form (see below). Overall, most lemma vs. leaf differences occurred in the mature peptide domain (8 of 47 amino acids), as previously noted.

Transformation of Barley and *E coli* with *Hth1*

Particle bombardment was used to transform barley with a full-length *Hth1* cDNA clone. This yielded 106 transformed plants, confirmed by PCR and Southern blot analysis (data not shown). All transformants resulted from three independent events, and all had multiple insertions of the transgene. RT-PCR, in which a downstream NOS primer was used, confirmed that the mRNAs detected were from the transgene and not from endogenous leaf-specific thionin sequences (see Methods; data not shown). Northern blot analysis detected little or no *Hth1* mRNA in leaves of transformants (Figure 3, A series). Therefore, alteration of *Hth1* was needed to improve expression.

Transformation in the pET32/*E. coli* system was also attempted in order to make soluble Trx (thioredoxin)-HTH1 fusion protein for antibody production. However, expression of the introduced *Hth1* gene was again weak (Figure 4A, lanes 1 and 2). After induction with IPTG, little *Hth1* mRNA and no Trx-HTH1 protein was produced. It was proposed (by JF) that the close proximity of the two 5′ methionine codons may interfere with translation. Removal of the sequence upstream from the second methionine codon (producing clone *Hth1Δ*) had a strong effect on expression. Previously, *Hth1* mRNA was almost undetectable until IPTG was added to the culture medium. However, *Hth1Δ* was constitutively expressed, and addition of IPTG caused substantial increases in mRNA levels. Most importantly, soluble HTH1 protein was produced (Figure 4B). The *trx-Hth1* and *trx-Hth1Δ* fusion transcripts were approximately the predicted transcript lengths of 1121 and 1071 b, respectively.

Antibodies from the purified Trx-HTH1Δ fusion protein had a high titre against thioredoxin and little or no titre against HTH1. However, stronger antibodies were successfully produced from a synthetic peptide representing the C-terminal 28 amino acids of the αHTH1 mature peptide (see Figure 2). This sequence contained all 6 amino acids not shared between α and βHTHs.

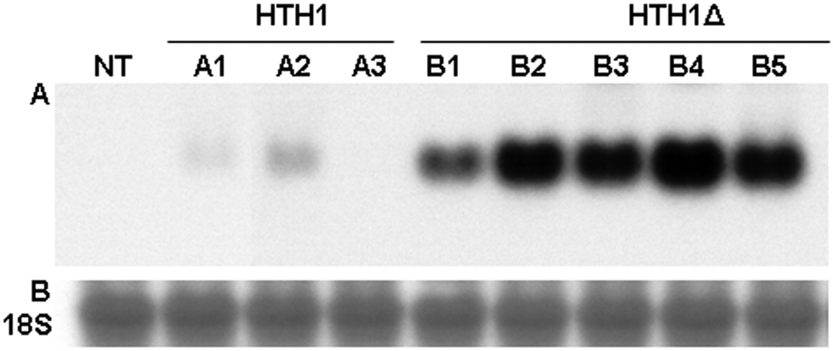


Figure 3. Hth1 mRNAs in barley transformed with Hth1 or Hth1Δ. Northern blot showing the effect of neighboring methionine codons on Hth1 transgene expression in transformed barley. Each lane contained 15 µg of leaf total RNA. ***Panel*** *A: Blot hybridized with ^{32}P-labeled Hth1 cDNA.* ***Panel B:*** *The blot in A was stripped and rehybridized with a leaf total RNA probe. NT, non-transformed control Golden Promise. A1 to A3 are among the Hth1/pAHC transformants with the highest Hth1 mRNA levels. B1 to B5 are among the Hth1Δ/pAHC transgenic lines with the highest Hth1 mRNA levels.*

Transformation of Barley with an Altered *Hth1*

These data indicated that the 5′ coding sequence of *Hth1* is inefficiently transcribed and translated. The same 5′ deletion of *Hth1* was produced so that *Hth1Δ* could be expressed from a plant transformation vector pAHC25 (Figure 5). Whatever the mechanism by which *αHth1Δ* expression was enhanced in *E. coli*, a positive effect was also seen in barley. Transformation of barley utilizing *Hth1Δ* produced over 169 plants from 5 independent insertion events (data not shown). In contrast to *Hth1* transformants, the leaves of all *Hth1Δ* transformants had high levels of *Hth1Δ* mRNA (Figure 3, B series). Comparison with untransformed Golden Promise RNA showed that there is no background signal from cross-hybridization with leaf-specific thionin mRNAs (Figure 3, lane NT). Although high levels of *Hth1Δ* mRNA were produced, HTH1 protein was still not produced in leaves or lemmas. We did not analyze developing seeds of barley transformants for HTH synthesis, since the same protein is normally produced in the endosperms of developing seeds. HTH1 protein from the expressed transgene would be indistinguishable from the HTH1 already present.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

Analysis of *αHthΔ* mRNA Translation

Since no HTH1 protein was detected in leaves, polyribosome analysis was conducted to determine whether the mRNA was translated. Total leaf polyribosomes were fractionated into size classes on sucrose gradients (Figure 6A). The fractions were analyzed on northern blots to determine whether translation along the *Hth1Δ* mRNA was blocked at any point. Most of the *Hth1Δ* mRNA occurred in the 80S fraction, indicating that, although initiation occurred, translation was very slow or blocked. However, almost half of the mRNA was distributed from the dimer through the 14-mer fractions, indicating that complete translation of the coding sequence probably occurred to some extent (Figure 6B). In the two transformed lines examined, B01 and B03, most of the *Hth1Δ* mRNA was associated with membrane-bound polyribosomes (Figures 6C and D), as previously found (*6*). Although 6 amino acids were deleted from the full-length signal peptide, the remaining 18-amino acid signal peptide was sufficient to initiate targeting HTH to the endomembrane system. Thus, expression of *Hth1* outside of the endosperm appears to be prevented by a 5′sequence that limits mRNA production or stability and by a blockage of translation.

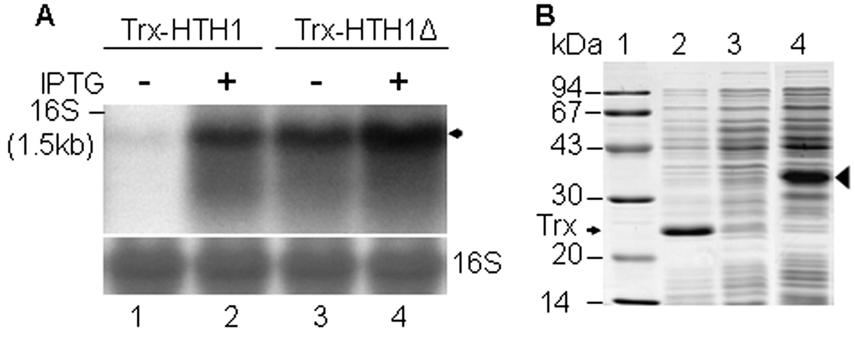


Figure 4. Expression of Trx-Hth fusion protein genes in E. coli expression vector pET-32b(+). ***Panel A, top****: Northern blot of total RNA from transformed E. coli BL21. The blot was probed with ^{32}P-labeled Hth1 cDNA. Lanes 1 and 2: RNAs from uninduced (-) and IPTG-induced (+), respectively, bacteria harboring Trx-Hth1/pET. Lanes 3 and 4: RNAs from the uninduced and induced bacteria harboring Trx-Hth1Δ/pET.* ***Panel A, bottom:*** *The stripped blot was rehybridized with a total RNA cDNA probe.* ***Panel B.*** *Separation of soluble bacterial proteins by SDS-PAGE. Bacteria from the induced cultures fractionated into soluble supernatant proteins and inclusion bodies. Lane 1, molecular mass standards. Lane 2, proteins from bacteria harboring empty vector Trx/pET (arrow - TRX protein). Lane 3, proteins from bacteria harboring Trx-Hth1/pET. Lane 4, proteins from bacteria harboring Trx-Hth1Δ/pET (wedge - Trx-HTH fusion protein).*

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

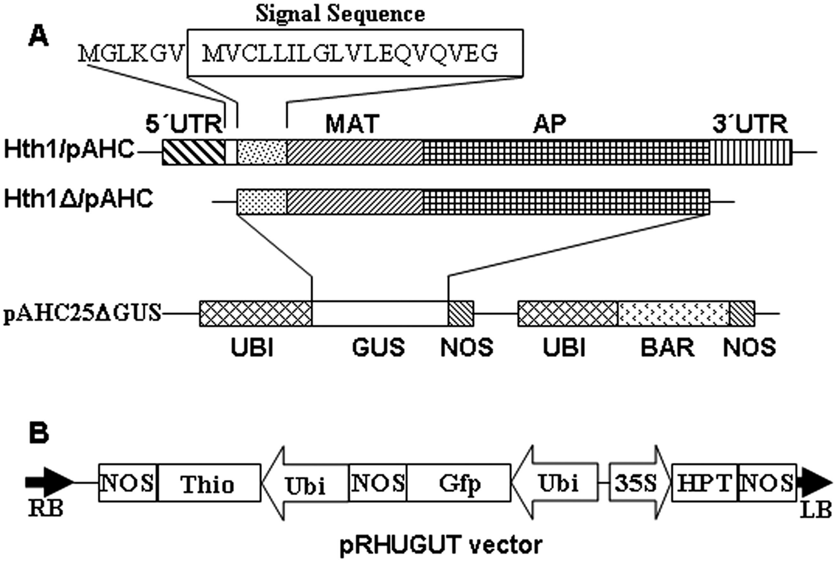


Figure 5. Expression vectors for stable transformation with seed-specific Hth1 and Hth1Δ genes and lemma-specific Lemthio1 gene. ***Panel A:*** *Transformation by particle bombardment. Hth1 represents the full-length cDNA; Hth1Δ represents Hth1 from which the sequence upstream of the second 5' methionine codon was deleted (MGLKGV). Hth1 and Hth1Δ were fused behind the ubiquitin promoter in pAHC25 (35), after removal of the GUS gene, creating Hth1/pAHC and Hth1Δ/pAHC, respectively. UBI, ubiquitin promoter; NOS, nopaline synthase termination sequence. BAR, phosphinothricin acetyltransferase gene.* ***Panel B:*** *Agrobacterium tumefasciens transformation vector. The pRHUGUT vector contains the Lemthio1 gene, constitutively expressed from the Ubi promoter. HPT, hygromycin resistance gene; 35S, tobacco mosaic virus 35S promoter; RB and LB, right and left Agrobacterium T-DNA borders; Gfp, green fluorescent protein coding sequence.*

Transformation of Barley with a *βHth*, *Lemthio1*

As a final approach, we undertook the transformation of barley with a lemma thionin gene, *Lemthio1*. This type II βHth cDNA had a greater chance to express well, since it would be re-expressed in its organ of origin. Transformation using the *Agrobacterium tumefaciens* method produced transformants with high levels of *Lemthio1* mRNA in the lemmas (Figure 7A). Unexpectedly, Golden Promise untransformed controls (GP) had no detectable *Lemthio1* on northern blots. Western blot analysis of lemma protein from the same transformed lines (C and F) showed that moderate levels of HTH1 protein were produced (Figure 7B). LEMTHIO1 was extracted with SDS, but only trace amounts could be extracted with Tris buffer. This indicated that LEMTHIO1 was associated with the cell wall. Three-fourths of the lemma cDNA clones had the identical sequence, suggesting a function for this protein in fibrous organs, such as the lemma.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

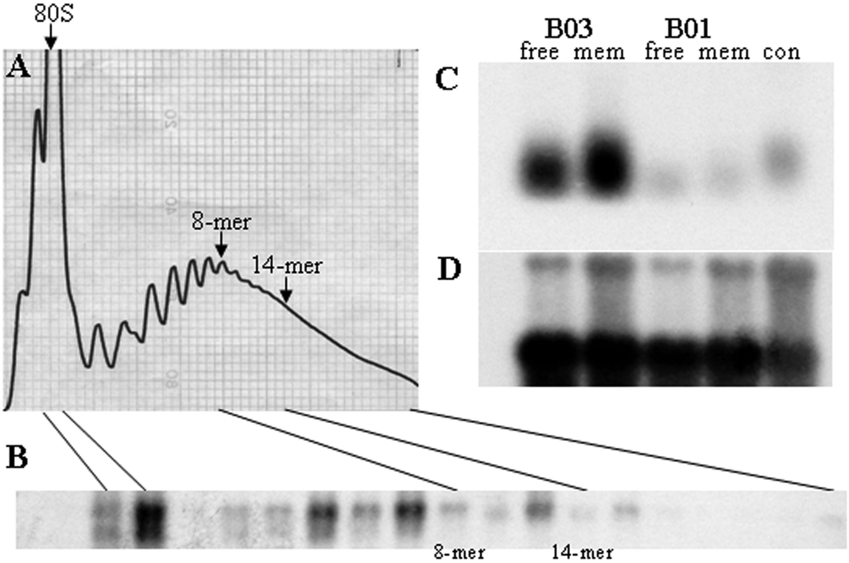


Figure 6. Analysis of Hth1Δ mRNA translation. ***Panel A:*** *Leaf polyribosomes from transformant B03 were separated on a sucrose density gradient, and the A_{254} was monitored as the gradient was fractionated. An "8-mer" refers to mRNA with 8 ribosomes attached.* ***Panel B:*** *Equal volume fractions were collected, and anayzed on a northern blot probed with ^{32}P-dCTP labeled Hth1 cDNA.* ***Panel C:*** *RNA from free and membrane-bound (mem) polyribosomes from leaves of lines B01 and B03 were northern blotted and probed as above. Control lane (con) is total leaf RNA from line B03.* ***Panel D:*** *Blot from panel C was stripped and reprobed with labeled total RNA cDNA, as above. Dark band is 18S rRNA; light upper band is 25S rRNA.*

Discussion

The α and β HTH peptides are both lethal to *F. graminearum* at concentrations that can be attained through genetic transformation. The endosperm-specific αHTH1 is more lethal by several-fold, but there appear to be constraints on expressing this gene outside of the endosperm. Given it's effectiveness, it is worthwhile to determine how this can be overcome.

Possible Effects of 5′ Methionine Codon Crowding

These studies show that the 5′ sequence of *Hth1* has pronounced effects on expression in systems as diverse as *E. coli* and barley. *Hth1* has two methionine codons near the 5′end. Removal of the first methionine from the leader sequence (producing *Hth1Δ*) enhanced *Hth1* gene expression. In *E. coli*, *HthΔ* mRNA

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

increased greatly, and HTH1 protein synthesis increased from a trace to high levels. In transformed barley, the enhancement was confined to a sharp increase in mRNA levels. Again, even though high mRNA levels were achieved by transformation with *Hth1Δ*, HTH protein was not produced in leaves. Florack *et al.* (*16*) transformed tobacco with the barley seed-specific *Hth-1* gene and produced HTH1 in leaves by combining a barley leaf thionin signal sequence with sequences encoding the HTH1 mature protein and acidic protein domains. The leaf signal sequence may ensure that HTH1 is deposited in a subcellular compartment where it may escape proteolysis. Although the protein bodies of the endosperm are analogous to vacuoles, the partial signal sequence of *Hth1Δ* may have been insufficient to be recognized by leaf vacuoles, even though *Hth1Δ* mRNA occurred on membrane-bound polyribosomes. Alternatively, the endoplasmic reticulum in lemmas may lack the appropriate signal recognition particle to interact with what may be an endosperm-specific signal sequence.

Regulation at the 5′ end of transcripts is quite complex and varied. The binding of regulatory proteins to the 5′region of certain mRNAs greatly complicates translation. In amaranth, *rbc*L mRNA is only translated in the light. McCormac et al. (*19*) identified a protein, p47, which binds the 5′UTR and 5′coding region (-66 to +60) of *rbc*L mRNA only in the light, suggesting that p47 may be involved in translation initiation. Translation of *opaque-2* mRNA in maize is inhibited by upstream open reading frame sequences, a feature found in mRNAs of other regulatory proteins and transcription factors (*20*). Translational regulation of expression by 5′ portions of plant virus transcripts has also been well documented (reviewed in Gallie, (*21*)). A sequence element in the 5′ UTR of pea *Lhcb1* is known to destabilize the mRNA in leaves but not in apical buds (*22*). In pea leaves, exposure to light causes polyribosomes to dissociate from a sequence in the 5′ UTR and 5′ coding region of *Fed-1* mRNA (*23*). Likewise, the 5′ UTR of the *Chlamydomonas* chloroplast *rbc*L gene destabilizes GUS transcripts in the light (*24*). Both the 5′UTR and coding region of the *Pet*E gene are required to regulate mRNA steady-state level in transgenic plants (*25*). Sequences in the 3′ UTR of plant mRNAs are known to influence mRNA stability (*26*).

Our studies could not distinguish between the various causes of failure to produce HTH1 protein in vegetative tissues. However, the use of our *Hth1* to transform oats (*27*) confirmed that the *Hth1* coding sequence was translatable. As in barley, *Hth1* mRNA was produced in leaves and endosperms. However, HTH1 protein was only produced in oat endosperm, showing that translation products from this mRNA could only accumulate in the cellular environment of the endosperm. We did not analyze developing seeds of barley transformants for HTH1 synthesis, since the same protein is produced in abundance in the endosperms of untransformed barley. The expressed gene would be indistinguishable from the α HTH1 already present. Oats do not present this immunological background problem. They have no mRNAs that cross-hybridize on northern blots with *Hth1*, and HTH1 antibodies do not cross-react with oat seed proteins (data not shown). This shows that *Hth1* lacks an as yet unidentified factor required for translation in vegetative tissues; alternatively, these tissues may actively inhibit translation. In some tissues, pre-translational controls may also be involved. In lemmas, *Lemthio1* mRNA was not detected on northern blots.

The *Hth-1* gene promoter contains at least 9 silencer motifs identical or similar to those identified in other genes (*28*). These negative elements may be involved in silencing *Hth1* gene expression in vegetative tissues, particularly the lemma.

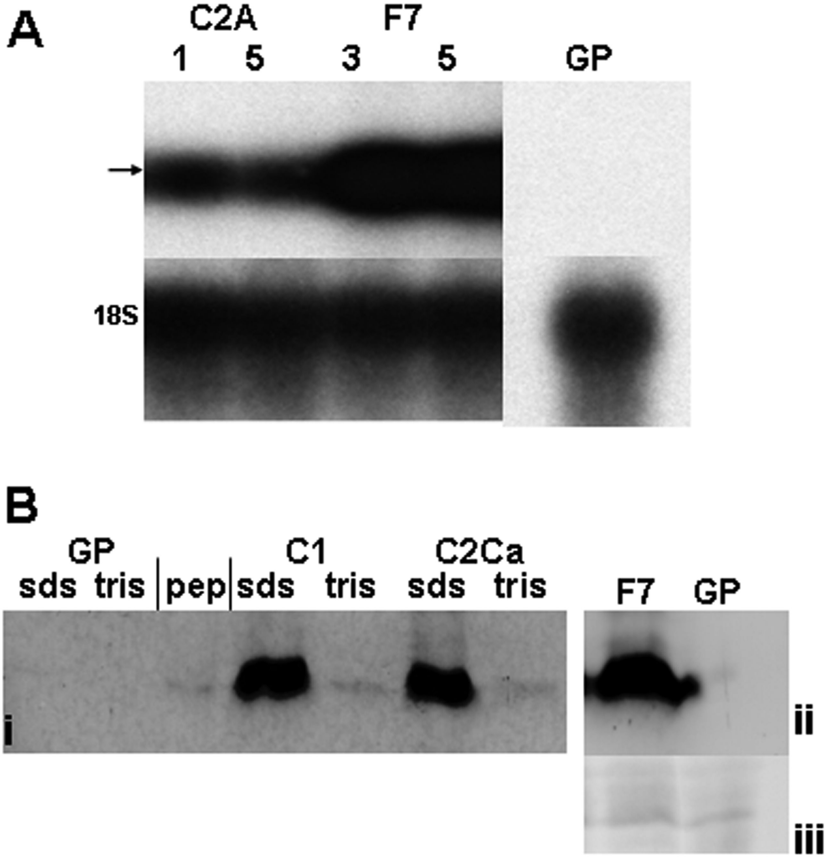


Figure 7. Over-expression of Lemthio1 gene in barley lemmas. Lemthio1 was cloned from lemmas, expressed from the pRHUGUT vector and transformed via A. tumefasciens. ***Panel A****: Northern blot showing high levels of Lemthio1 mRNA (arrow) in lemmas of T_o plants. C2A, 1 and 5, and F7, 3 and 5, are from transgenic events C and F. GP, untransformed Golden Promise. Lower panels: Blots reprobed with 18S rRNA probe.* ***Panel B****: Western blots showing LEMTHIO1 protein levels in lemmas of T_0 transformants. Transformation events shown represent those with the highest expression levels produced by each expression vector.* ***i:*** *Bound LEMTHIO1 was released with SDS detergent, relative to Tris buffer in C events. pep - LEMTHIO1 synthetic peptide.* ***ii:*** *F event and GP control (GP has essentially undetectable LEMTHIO1 background.)* ***iii:*** *Ponceau red staining showing total protein (20 μg) loaded in each lane of the 14% SDS-PAGE gels; strips representing 17 to 30 kDa proteins are shown.*

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

The sequence context surrounding the start codon is known to affect post-transcriptional expression. In the Hth1/pAHC vector, *Hth1* has two nearby 5′ methionine codons. Generally, translation should start from the first AUG (met codon), unless "leaky scanning" occurs (*29*). The *Hth1* sequence contexts around each methionine codon are each favorable for translation. Thus, there is a purine at position -3 and a G at position +4 (*30*). The inhibition of HTH synthesis in leaves also could result from mRNA secondary structure. Secondary structure between the 5′ cap and the AUG codon inhibits translation initiation (*30*).

Translation Analysis

The attachment to membrane-bound polyribosomes indicated that translation initiation on *Hth1Δ* proceeded normally. Because the *Hth1Δ* mRNA in the barley transformants occurred on membrane-bound polyribosomes, translation must have proceeded through enough of the signal sequence to allow attachment to the ER. Ribosomes can occur on mRNA at maximal stacking densities covering 27 to 29 bases per ribosome (*31*). In the 384 b from the second methionine codon to the stop codon, a maximum of 14 ribosomes could bind the mRNA. The occurrence of a small but significant portion of *Hth1Δ* mRNA in larger 8- to 14-mer polyribosomes indicated that full translation occurred to some extent. In our study, roughly half of the *Hth1Δ* mRNA appeared in the monosome (80S) fraction, which could indicate stalling of translation (Figure 5B). However, the remainder of the mRNA appearred in the expected range of size classes. The two 5′ methionine codons could, theoretically, interfere with each other. The codons are only 15 b apart, while the center to center coverage of ribosomes is 28 b or greater. Obviously, this is overcome in the developing endosperm, where translation starts at the second methionine codon.

The successful transformation of barley using the lemma-specific *Lemthio1* thionin gene shows that *Hth* expression at the protein level can be achieved. For now, a constitutive promoter (*Ubi1*) must be used, although tissue-specific promoters are being developed. The key to success may be related to the signal peptide. The *Lemthio1* leader remained attached to its native transcript elements, and no attempts were made to link this leader to endosperm-specific *Hths*. Future research calls for attaching the *Lemthio1* leader sequence to the *Hth1* mature peptide and C terminal domains to determine whether the more potent HTH1 protein can be produced in lemmas. Presently the amount of LEMTHIO1 produced in lemmas of transformants appears to be substantial, so that a chance of fungal resistance seems reasonable.

Materials and Methods

Thionin Toxicity Plate Assays

Conidiospores of *F. graminearum* field strain NRRL29169, provided by Kerry O'Donnell (ARS, Peoria), were grown on potato dextrose agar. Assays were conducted in large well microtitre plates; each well contained 0.1 ml carrot juice, water, spores (1000 or 4000) and HTH, to a total volume of 0.5 ml. Plates were incubated on a gyratory shaker at 23° C for 48 h. Carrot juice was prepared by dicing 100 g of organic carrots into 500 ml deionized water and autoclaving.

cDNA Library Construction and Gene Cloning

Hth1 **-** For cloning of *Hth1*, developing endosperm tissue of barley cultivar "Morex" was collected, and total RNA was extracted (*32*). A cDNA library was constructed in λZAPII (Stratagene) and probed with oligonucleotide THIOA (5′-AAGGTTGTAGCAGTTTCTTCCTAGGGT) as described in Skadsen *et al.* (*33*). THIOA encodes a conserved amino acid sequence, TLGRNCYNL, near the N-terminus of the mature peptide. Helper phage were used to convert phagemids to pBluescript plasmids. The cDNA inserts were excised by digestion with EcoRI, and the longest of these (*Hth1*) was sequenced with Big Dye (Applied Biosystems). Sequences were determined by capillary electrophoresis by the University of Wisconsin Biotechnology Center.

Lemthio1 **-** Total RNA was purified (*34*) from lemmas of Morex barley when developing seed spikelets were in the milk to early dough stages. Polyadenylated mRNA was purified using oligo d(T) beads (GenElute, Sigma-Aldrich), and Superscript III reverse transcriptase was used to produce cDNA. Twelve type 2 leaf thionin GenBank accessions were aligned using PILEUP (GCG, Madison, WI; L36882, X05576, X05587, X05588, X05589, M19046, M19047, M19048, AJ508712, 11401356, 11401357, L36883). Two sets of up- and downstream conserved sequences from the alignment were used as PCR primers on both lemma and leaf cDNA templates: 1) LfThio1UP, 5'-CGGGCAGAAACTGCTACAAC and LfThio1DN, 5'-CCATCATTACAGAAACGGGCAC, 2) LfThio2UP, 5'-AGAAAGTGCTACAACACTTGCC and LfThio2DN, 5'-TCAACAGACTGAATGACTGCAC. PCR was conducted with Taq polymerase (Invitrogen). PCR was conducted for 35 cycles of denaturation (94°C, 30 sec), annealing (45 to 50°C) and extension (72°C, 2 min). Reactions included 5% (v/v) DMSO. Products were cloned into the TOPO TA vector (Invitrogen) and sequenced using BigDye (Applied BioSystems). RACE, using Superscript III polymerase, was used to determine the 5' and 3' end sequences of thionin transcripts (Invitrogen). A downstream primer (Thio5'RACE, 5'-GGCGAAGCGGCAGGTGTTGTAGCAG), overlapping the 5' end of the above PCR products, was used in a PCR reaction with the 5' GeneRacer primer/adaptor (Invitrogen) as the upstream primer; lemma cDNA was used as the template. Likewise, a downstream primer (Thio3'RACE, 5'-CATGTGCCCGTTTCTGTAATGATGGTGCAG), overlapping the 3' ends of the above PCR products, was used in a PCR reaction with the 3' GeneRacer primer. RACE reactions were conducted as per the supplier's instructions, using Superscript III polymerase (Invitrogen). After 5' UTR sequences were determined, an upstream primer corresponding to a 5'UTR sequence (ThioUP4, 5'-TCAATCCAACATAGCCATTTCTCATTCTTC) was synthesized and used in a PCR with downstream primer LFThio1DN, above. A 444 bp 5′ product was cloned into pCR2.1 (Invitrogen), producing Thio18. To produce an overlapping 3' sequence, a PCR was conducted using upstream primer LFThio1UP (above) and the 3' GeneRacer nested primer. The resulting overlapping 3' sequence

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

of 528 bp was cloned into pCR2.1, producing clone Thio41. Thio18 (5’) and Thio41 (3’) had overlapping identical sequences of 280 bp. A common PstI site occurred in the overlap region and was used to join both halves, resulting in the near full-length thionin clone Thio1841 (representing gene *LemThio1*, GenBank accession FJ026806).

Expression of a Thioredoxin-Thionin Fusion Gene in *E. coli*

Hth1 had two methionine codons at the 5′ coding end. The first codon occurred within an NcoI site, and a second NcoI site occurred 11 bp downstream from the stop codon. *Hth1* was excised with NcoI and ligated into the pET32b(+) vector (Novagen) at the NcoI site, producing a *thioredoxin-Hth1* fusion gene (*Trx-Hth1*/pET). This plasmid was used to transform *E. coli* strain BL21 (Novagen). For construct *Trx-Hth1Δ*/pET, the sequence from the second 5′ methionine through the stop codon was amplified by PCR using upstream primer THIOCC (5′-CGTGCCATGGTGTGTTTACTTATACT) and downstream primer THIOD (5′-TTTTCCATGGTTATTTGGGGAAGCCTGTA). Products were ligated into pET32b(+) at the NcoI site. Fusion genes were induced with 1 mM IPTG for 4 h. The fusion protein was extracted from BL21 cells and purified on His-Bind (Novagen).

HTH1 antibodies were produced in rabbits from a synthetic peptide representing amino acids 18 through 45 of the mature peptide (see Figure 2). Peptide synthesis and antibody production were performed by AnaSpec, Inc. (San Jose, CA). *E. coli* and barley proteins were separated by electrophoresis in 14% SDS-polyacrylamide gels, and HTH was detected by western blots, as described (*33*).

Transformation with *αHth1*, *αHthΔ*, and *Lemthio1*

Hth1 was excised from pBluescript with SacI and EcoRV. The *UidA* (GUS) gene was removed from the plant expression vector pAHC25 (*35*) by digestion with SacI and SmaI. *Hth1* was then ligated into pAHC25 between the *Ubi1* maize ubiquitin promoter and NOS termination sequence, producing Hth1/pAHC. For construction of a *Hth1Δ* expression vector, the cDNA sequence of *Hth1* from the second methionine codon through the stop codon was amplified by PCR. The PCR products were digested with Sma1 and Sac1. The 400 bp product was ligated at SmaI and SacI sites of pAHC25, after removing the GUS gene with a SmaI/SacI digest. The resulting construct was named *Hth1Δ*/pAHC (Figure 5A) and used for stable transformation.

Golden Promise barley seeds were grown in a greenhouse with supplemental lighting to provide a 16 h photoperiod. Immature embryos were transformed by particle bombardment, according to Wan and Lemaux (*36*). Embryo-derived calli were subcultured every 2 weeks and maintained in darkness at 24°C. Six weeks after bombardment, calli were transferred to regeneration media and were maintained under a 16 h photoperiod. Regenerants were transferred to rooting media, and those that developed strong root systems were transferred to pots and grown in a greenhouse.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

The Agrobacterium cloning vector pRHUGUT (Figure 5B) was constructed by combining *gfp* (green fluorescent protein) and *Lemthio1* expression modules. The constitutive *gfp* module (*Ubi1-gfp-nos*) was excised from pBluescript plasmid pACHsgfp (*37*) and joined with the constitutive *Lemthio1* module (*Ubi1-Lemthio1-nos*). The latter was produced by cloning *Lemthio1* in place of the *GUS* gene in pAHC25 (*35*). The modules were then inserted into the Cla and Spe sites in binary vector pRSHyg7 (*38*, *39*). Immature embryo explants from the Golden Promise cv. were transformed by co-cultivation with *Agrobacterium tumefaciens* strain AGL0 (*40*), harboring expression vector pRHUGUT. Transformation was conducted as described (*41*, *42*). Hygromycin was used for selection of calli, and GFP was used as a visual marker for selection of calli. Plantlets were regenerated on the selection agent for 6-8 weeks and then transferred to potting soil and grown to maturity.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

Genomic DNA Extraction, PCR, and DNA Blot Analyses

Genomic DNA was extracted from leaves of greenhouse-grown plants using the CTAB procedure (*43*). For detection of *Hth1* and *Hth1Δ* transgenes, PCR was conducted using an upstream primer, UBI2 (5′-CTC ACCCTGTTGTTTGGTGTTACTTCTGC), which primes near the 3′ end of the ubiquitin promoter. The downstream primer, NOS1 (5′-AATCATCGCAAGACCGGCAACAGGATTCA), which primes near the 5′ end of the NOS termination sequence. This pair of primers amplified the *Hth* and *gfp* genes simultaneously. Typical reactions contained 0.4 µM of each primer, 0.4 mM dNTPs, 500 0g genomic DNA, 10% (v/v) of DMSO and 2.5 U of Taq polymerase (Promega, Madison WI). The PCR program was conducted with 35 cycles of denaturing at 95°C for 30 sec, annealing at 50°C for 47 sec, and extension at 72 °C for 2 min. DNA from PCR-positive plants was used for a Southern blot analysis. The full *Hth1* cloned insert was excised from pBluescript, radiolabeled with ^{32}P-dCTP and used as a probe. Hybridization and washing were conducted at 60°C. Blotting, probe synthesis, hybridization and radiography were performed according to Skadsen *et al.* (*33*).

RNA and Polyribosome Analysis

Free and membrane-bound polyribosomes were prepared from leaves essentially according to Davies *et al.* (*44*). Polyribosomes were separated on 10 to 40% sucrose density gradients, fractionated and applied to northern blots (*45*). RNA was prepared by dissolving polyribosomes in 100 mM Tris, 100 mM NaCl, 20 mM EDTA, 20 mM EGTA, pH 9.0 and extracting in phenol/chloroform/isoamyl alcohol.

As an internal hybridization control, a total RNA probe was prepared (*46*). cDNA was prepared as above, using 1 µg leaf total RNA. Instead of an oligo d(T) primer, 8 ρmoles of random hexamers were added, along with ^{32}P-dCTP. Supplemental unlabeled dCTP was added after 12 min. After a 60 min reaction at 43°C, RNA was hydrolyzed with RNase for 30 min. Four µg of tRNA was added, and the labeled cDNA was precipitated with ethanol. Hybridization was conducted at 50°C using 10^5 dpm per ml of hybridization solution. High stringency northern blot hybridizations of *Hth* sequences were conducted (*33*).

Acknowledgments

This research was conducted at the USDA-ARS Cereal Crops Research Unit in Madison, WI. We thank John Herbst and Laura Oesterle for excellent technical support and Berne Jones (USDA/ARS/CCRU, Madison, WI) for providing purified barley endosperm HTH. pAHC25 was a generous gift from Peter Quail, USDA/ARS Plant Gene Expression Center, Albany, CA). This work was supported by the U.S. Department of Agriculture (Project 3655-21430-006-05S) and Univ. of Wisconsin SCA 58-3655-4-148 in a cooperative project (ID-0405-SK-038) with the U.S. Wheat and Barley Scab Initiative. Early funding was provided by the American Malting Barley Association. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the USDA. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the USDA. or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

References

1. Redman, G. D.; Fisher, N. *J. Sci. Food Agric.* **1969**, *20*, 427–432.
2. Rodriguez-Palenzuela, P.; Pintor-Toro, J.; Carbonero, P.; Garcia-Olmedo, F. *Gene* **1988**, *70*, 271–281.
3. Stec, B. *Cell Mol. Life Sci.* **2006**, *63*, 1370–1380.
4. Han, K-H.; Park, K-H.; Yoo, H-J.; Cha, H.; Suh, S. W.; Thomas, F.; Moon, T-S.; Kim, S-M. *Biochem. J.* **1996**, *313*, 885–892.
5. Bohlmann, H.; Clausen, S.; Behnke, S.; Giese, H.; Hiller, C.; Reimann-Philipp, U.; Schrader, G.; Barkholt, V.; Apel, K. *EMBO J.* **1988**, *7*, 1559–1565.
6. Ponz, F.; Paz-Ares, J.; Hernandez-Lucas, C.; Carbonero, P.; Garcia-Olmedo, F. *EMBO J.* **1983**, *2*, 1035–1040.
7. Hernandez-Lucas, C.; Royo, J.; Paz-Ares, J.; Ponz, F.; Garcia-Olmedo, F.; Carbonero, P. *FEBS Lett.* **1986**, *200*, 103–106.
8. Florack, D. E. A.; Stiekema, W. J. *Plant Mol. Biol.* **1994**, *26*, 25–37.
9. Carmona, M.; Hernadez-Lucas, C.; Martín, C.; González; Garcia-Olmedo, F. *Protoplasma* **1993**, *173*, 1–7.
10. Carbonero, P.; Garcia-Olmedo, F.; Hernandez-Lucas, C. *J. Agric. Food Chem.* **1980**, *28*, 399–402.
11. Reimann-Philipp, U.; Schrader, G.; Marinoia, E.; Barkholt, V.; Apel, K. *J. Biol. Chem.* **1989**, *264*, 8978–8984.
12. Gausing, K. *Planta* **1987**, *171*, 241–246.
13. Carmona, M. J.; Molina, A.; Fernandez, J. A.; Lopez-Fando, J. J.; Garcia-Olmedo, F. *Plant J.* **1993**, *3*, 457–462.

14. Epple, P.; Apel, K.; Bohlmann, H. *Plant Cell* **1997**, *9*, 509–520.
15. Charity, J. A.; Hughes, P.; Anderson, M. A.; Bittisnich, D. J.; Whitecross, M.; Higgins, T. J. B. *Funct. Plant Biol.* **2005**, *32*, 35–44.
16. Florack, D. E. A.; Dirske, W. G.; Visser, B.; Heidekamp, F.; Stiekema, W. J. *Plant Mol. Biol.* **1994**, *24*, 83–96.
17. Evans, J.; Wang, Y.; Shaw, K.-P.; Verdon, L. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5849–5853.
18. Skadsen, R. W.; Hohn, T. M. *Physiol. Mol. Plant Pathol.* **2004**, *64*, 45–53.
19. McCormac, D. J.; Litz, H.; Wang, J.; Gollnick, P. D.; Berry, J. O. *J. Biol. Chem.* **2001**, *276*, 3476–3483.
20. Lohmer, S.; Maddaloni, M.; Motto, M.; Salamini, F.; Thompson, R. D. *Plant Cell* **1993**, *5*, 65–73.
21. Gallie, D. R. *Plant Mol. Biol.* **1993**, *44*, 77–105.
22. Anderson, M. B.; Folta, K.; Warpeha, K. M.; Gibbons, J.; Gao, J.; Kaufman, L. S. *Plant Cell* **1999**, *11*, 1579–1589.
23. Dickey, L. F.; Nguyen, T-T.; Allen, G. C.; Thompson, W. F. *Plant Cell* **1994**, *6*, 1171–1176.
24. Salvador, M. L.; Klein, U.; Bogorad, L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1556–1560.
25. Halliwell, C. A.; Webster, C. I.; Gray, J. C. *Plant J.* **1997**, *12*, 499–506.
26. Green, P. J. *Plant Physiol.* **1993**, *102*, 1065–1070.
27. Carlson, A.; Skadsen, R. W.; Kaeppler, H. F. *In Vitro Cell. Dev. Biol.: Plant* **2006**, *42*, 318–323.
28. Fernández, J. A.; Moreno, M.; Carmona, M. J.; Castagnaro, A.; Garcia-Olmedo, F. *Biochem. Biophys. Acta* **1993**, *1172*, 346–348.
29. Kozak, M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2662–2666.
30. Kozak, M. *J. Biol. Chem.* **1991**, *266*, 19867–19870.
31. Wolin, S.; Walter, P. *EMBO J.* **1988**, *7*, 3559–3569.
32. Skadsen, R. W. *Plant Physiol.* **1993**, *102*, 195–203.
33. Skadsen, R. W.; Sathish, P.; Kaeppler, F. H. *Plant Sci.* **2000**, *156*, 11–22.
34. Chirgwin, J. M.; Prybyla, A. E.; MacDonald, R. J.; Rutter, W. J. *Biochemistry* **1979**, *18*, 5294−5299.
35. Christensen, H. A.; Quail, H. P. *Transgenic Res.* **1996**, *5*, 213–218.
36. Wan, Y.; Lemaux, P. G. *Plant Physiol.* **1994**, *104*, 37–48.
37. Kaeppler, H. F.; Menon, G. K.; Skadsen, R. W.; Nuutila, A. M.; Carlson, A. R. *Plant Cell Rep.* **2000**, *19*, 661–666.
38. Wang, M-B.; Li, Z-Y.; Matthews, P. R.; Upadhyaya, N. M.; Waterhouse, P. M. *Acta Hort.* **1998**, *461*, 401–407.
39. Xue, G. P.; Patel, M.; Johnson, J. S.; Smyth, D. J. *Plant Cell Rep.* **2000**, *21*, 1088–1094.
40. Lazo, G. R.; Stein, P. A.; Ludwig, R. A. *Biotechology* **1991**, *9*, 963–967.
41. Tingay, S.; Mc Elroy, E.; Kalla, R.; Fieg, S.; Wang, M.-B.; Thornton, S.; Brettell, R. *Plant J.* **1997**, *11*, 1369–1376.
42. Patel, M.; Johnson, J. S.; Brettell, R. I. S.; Jacobsen, J.; Xue, G. P. *Mol. Breed.* **2000**, *6*, 113–123.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

43. Dellaporta, S. L.; Wood, J.; Hicks, J. B. In *Molecular Biology of Plants: A Laboratory Course Manual*; Malmberg, R., Messing, J., Sussex, I., Eds.; Cold Spring Harbor Press, New York, 1985; pp 36−37.
44. Davies, E.; Larkins, B. A.; Knight, R. H. *Plant Physiol.* **1972**, *50*, 581–584.
45. Skadsen, R. W.; Scandalios, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2785–2789.
46. Federico, M. L.; Kaeppler, H. F.; Skadsen, R. W. *Plant Mol. Biol.* **2005**, *57*, 35–51.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch017

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

Chapter 18

Expression of Designed Antimicrobial Peptides in *Theobroma cacao* L. Trees Reduces Leaf Necrosis Caused by *Phytophthora* spp.

Luis C. Mejía,[1,2] Mark J. Guiltinan,[1] Zi Shi,[1] Lena Landherr,[1] and Siela N. Maximova*,[1]

[1]The Department of Horticulture and The Huck Institutes of the Life Sciences, The Pennsylvania State University, 421 Life Sciences Building, University Park, PA 16802, U.S.A.
[2]Current address: Smithsonian Tropical Research Institute, Apartado 0843-03092 Balboa, Ancon, Republic of Panama
***E-mail: snm104@psu.edu**

Antimicrobial peptides naturally occur in a wide range of life forms including bacteria, fungi, plants and animals and represent an important component of their innate immunity systems. These peptides can inhibit the growth of animal and plant pathogenic bacteria and fungi. Designed synthetic peptides based on the structure of naturally occurring peptides have been shown to inhibit the growth of animal and plant pathogenic bacteria and fungi. In this chapter we report the engineering of *T. cacao* plants to express synthetic peptides and the *in planta* effects on the damage (necrosis) caused by two important oomycete pathogens of this crop. *Theobroma cacao* transgenic lines expressing synthetic peptides D5C and pD4E1 exhibited less foliar damage by pathogen *Phytophthora capsici* and *P. palmivora* than wild type cacao. We discuss the potential effects of antimicrobial peptides on non-pathogenic symbiotic microorganisms of plants e.g. mychorrizae and endophytes.

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

Introduction

Theobroma cacao is a tree crop cultivated throughout the tropics primarily for harvesting of its seeds, which are the source of cocoa powder, butter, and liquor. The World Cocoa Foundation estimates the current annual production of cocoa at 3 million tons per year and to have a market value of $ 5.1 billions (*1*). However world production of cocoa is severely affected by biotic diseases, which represent the most limiting factor to this crop. The following five diseases account for 20% reduction of cacao production: Black pod caused by oomycete species in the genus *Phytophthora*, including *P. capsici* and *P. palmivora*, the fungal diseases Witches' broom, Frosty pod and Vascular streak dieback caused by *Moniliophthora perniciosa*, *Moniliophthora rorei* and *Oncobasidium theobromae* respectively, and swollen shoot caused by cacao swollen shoot virus (*2*). Due to its occurrence in all countries producing cacao and because of its destructive capacity, Black pod is the most important disease of this crop and effective long-term control measures for it have not been developed. In general, the methods for controlling diseases of cacao include chemical control by means of fungicides and inducers of plant defense mechanisms, phytosanitation methods such as removal and burial of diseased pods, biological control with antagonistic microbial endophytes and epiphytes, and the breeding and employment of varieties with genetic resistance to pathogens. Of these, genetic resistance is the most promising approach, although sources of resistance genes for some of these diseases have thus far provided only partial resistance.

Transgenic strategies for improving the resistance of cacao to plant pathogens, have been explored in only one study to date (*3*). In that study, transgenic cacao plants engineered to over-express a class I chitinase gene (*4*) showed marked reduction of foliar necrosis caused by the fungal pathogen *Colletotrichum gloeosporioides*. Encouraged by the success of that study, we engineered cacao plants to express high levels of synthetic peptides with known broad antimicrobial activities to explore that possibility of reducing the damage caused by pathogens. Antimicrobial peptides are important defense molecules in the innate immune responses of multi-cellular organisms and have been reported from bacteria, plants, fungi, and animals (*5*, *6*) and as fully reported elsewhere in this book. Several designed synthetic antimicrobial peptides analogs have been shown to inhibit the growth of a wide range of plant pathogens (*7*, *8*). The synthetic peptides used in this study were designed by Dr. Jesse M. Jaynes and were previously reported to increase resistance to microbial pathogens in transgenic plants (US Patent # 5597945).

We report here on the regeneration, development and analysis of transgenic cacao plants engineered to express several antimicrobial peptides. The results of our *in vivo* bioassays with purified peptides and with transgenic cacao leaves indicated that the peptides have a strong efficacy against 3 important cacao oomycete pathogens.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

Materials and Methods

Transformation Vector Construction and Generation of Transgenic Lines

Gene cassettes encoding for synthetic antimicrobial peptides *CaMV 35S-D5C (cyto)-nos*, *CaMV 35S-D5C (ER-retained)-nos* and *CaMV 35S -D4E1-nos* were obtained from Demegen, Inc. (www.demegen.com) and each individually ligated as a blunt end fragments into the Spe1 site of pGH00.0131 binary vector (derived from vector pGH00.0126) (*9*) containing *EGFP* (Clontech, Palo Alto, CA) and *NPTII* (*10*) marker genes both under the control of high level constitutive *E12-Ω CaMV 35S* promoter (*11*). The resulting binary vectors (pGM021023-D5C-cyto; pGM020801-D5C-ER-retained and pGM021123-D4E1) were introduced into the disarmed *Agrobacterium tumefaciens* strain AGL1 (*12*) by electroporation (*13*) (Table I).

Table I. Transgenic cacao tree lines containing genes encoding for antimicrobial peptides

Line ID	*Binary Vector*	*Peptide Gene*
31	pGH00.0126	EGFP control, no peptide
70	pGM02.1023	D5C cytopeptide
71	pGM02.0801	D5C ER retained
74	pGM02.1023	D5C cytopeptide
76	pGP02.1122	D4E1
77	pGM02.0801	D5C ER retained

Plant transformations were performed as previously described (*3*, *9*, *14*). In brief, staminodes dissected from immature cacao flowers of genotype PSU-Sca6 were cultured to produce somatic embryos by methods previously described (*15*, *16*). Cotyledons from primary somatic embryos were co-cultivated with *Agrobacterium tumefaciens* (AGL1) containing individual binary plasmids (described above) and further cultured on 50 mg/l geneticin selection to produce secondary embryos. Transgenic secondary somatic embryos were selected based on green fluorescence due to expression of the *EGFP* marker gene (Clontech, Palo Alto, CA). Five stable transgenic lines expressing different synthetic peptides were generated (Table I). Control transgenic lines were regenerated after transformation with pGH00.0126. Additionally control PSU-Sca6 plants were regenerated by somatic embryogenesis (*16*) and used as non-transformed controls.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

Fluorescent Imaging of GFP

Unopened flowers and mature leaves were collected from greenhouse grown transgenic and control non-transgenic PSU-Sca6 trees. Fluorescent EGFP images were obtained as previously described (*9*). Images were captured using Nikon SMZ-4 dissecting microscope equipped with an epi-fluorescence attachment, a 100W mercury light source and a Nikon D90 digital SLR camera. The microscope filters used were 520-560 nm emission filter and 450-490 nm excitation filter.

Genomic PCR Analysis

Analysis was performed as previously described (*9*) Gene specific primer pairs were designed to amplify fragments from *EGFP* transgene (630bp), cacao actin gene (Tc01g010900, 566 bp), and *D5C-cyto* transgene (87 bp) (Table II). The primers used to verify the incorporation of *D4E1* included forward *EGFP* primer and reversed D4E1 primer amplifying fragment size of 1758 bp (Table II).

Leaf genomic leaf DNA from fully developed transgenic plants from independent transgenic lines 31, 70, 74 and 76 (Table I) and non-transgenic PSU-Sca6 plants were prepared as previously described (*3*). One µl of genomic DNA (5ng/ul) were added to each individual PCR reaction mix. Each PCR reaction also contained 10 µl Sigma JumpStart REDTaq ReadyMix reaction mix and both forward and reverse primers at final concentration 0.25 µM in final volume of 20µl. Reactions were prepared at 4°C.

Individual control PCR reactions were also performed with plasmid DNA from vector pGH00.0126, pGM02.1023 and pGP02.1122. Plasmid DNA was isolated using the Promega Wizard Plus Minipreps DNA Purification System (A7100, Promega Co., Madison, WI). Plasmid DNA templates was prepared via dilution series with DNA from salmon testes (Sigma-Aldrich Co., St. Louis, MO, #D-1626). Total of 0.15 pg of the respective plasmid DNA was added to each control PCR reaction. This represents an equal molar amount of plasmid DNA compared to the *EGFP* DNA contained in 5 ng total cacao genomic DNA present in the leaf extract, assuming single copy/insertion of the *EGFP* gene. Reactions were prepared at 4°C as Sigma JumpStart REDTaq ReadyMix reaction mix and primers were added as described above.

PCR conditions for all reactions were: 94°C for 2 min, then 35 cycles of 94°C for 45 sec., 55°C for 45 sec, 72°C for 1 min. The final cycle was followed by incubation at 72°C for 7 min. Ten µl of each PCR reaction were loaded onto 2% resolution agarose gel (IBI Scientific) for electrophoresis.

Semiquantitative RT-PCR of Cacao Tissues

Total RNA was isolated as previously described (*17*) from leaves at developmental stage C (Figure 1), collected from control non-transform PSU-Sca6 and transgenic plants from lines 31, 70, 74 and 76 as described in Table I. For each control and transgenic line, three biological replicates were collected and analyzed. Cacao cDNA was synthesized in a final volume of 25µl from 2µg of total cacao RNA using M-MLV reverse transcriptase (New England Biolabs,

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

Inc., Ipswich, MA). RNA and 0.5μg oligodT were added into sterile water to the volume of 18μl, the mixture was incubated at 70°C for 5min and chilled on ice, which was further treated by adding 10x reverse transcription buffer (New England Biolabs, Inc., Ipswich, MA), 0.1M fresh made DTT and 10mM dNTP. The mixture was held at 42°C for 2min, cDNA synthesis was conducted by incubating with 10 units of reverse transcriptase MMLV (New England Biolabs, Inc., Ipswich, MA) at 42°C for 1hr and the reaction was terminated at 70°C for 15min. Semi-quantitative RT-PCR was performed using gene specific primers (Table II, same as primers used for genomic PCR) to assay the levels of gene expression of *TcActin*, *EGFP D5C-cyto* and *D4E1*. RT-PCR was performed use 1 μl of ½ diluted cDNA and 5μM of the primers described above. Titration of cycles was carried out to find the linear amplification stage. It was determined that the PCR of *EGFP*, *D5C-cyto* and *D4E1* was within its linear range at 26 cycles using the following condition: 94°C for 30 sec., 55 °C for 30 sec, 72°C for 1 min. Similarly, PCR of control *TcActin* was performed under non-saturating condition to keep the reactions within the linear range (26 cycles at 94°C for 30 sec., 55 °C for 30 sec, 72°C for 1 min). The PCR products were analyzed on 1% agarose gel, stained with ethidium bromide and the expression values of the transgenes and *TcActin* were quantified use ImageQuant software (Molecular Dynamics, Amersham Bioscience) (*18*). Relative expression values of the transgenes were calculated by dividing the intensity values of individual target genes to the intensity value of *TcActin*.

Table II. Primer sequences of transgenes and native cacao genes utilized to genomic and semi-quantitative PCR analysis

Gene Name	***Forward Primer Sequence***	***Reverse Primer Sequence***
EGFP	5′-GTA AAC GGC CAC AAG TTC AGC GT -3′	5′-TTA CTT GTA CAG CTC GTC CAT GCC- 3′
TcActin	5′-TGG TGT CAT GGT TGG NATG-3′	5′-GGC ACA GTG TGA GAN ACA CC-3′
D5C-Cyto	5′-ATG AAG AGG AAG CGT GCA GTT-3′	5′-TTA GAA AGC CAC ACC GAG CCT-3′
D4E1	5′-GGC GGA TCC TTT AAG TTG AGA GCT-3′	5′-AAG CTT GCA TGC CTG CAG CAG-3′

Leaf Disk Bioassay of Antifungal Activity of Transgenic Cacao Plants against Two *Phytophthora* Species

Cacao leaf development proceeds through successive stages we have categorized as stages A-E (Figure 1). In preliminary work, we established that leaves in development stage C are appropriate for this bioassay to retaining their

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

viability and green color for 7 days after harvest, but are sufficiently soft to allow pathogen infection. Large fully expanded light green and soft cacao leaves (stage C) were collected from greenhouse-grown transgenic and control cacao plants propagated via rooted cuttings and grown for minimum of 4 years. Each leaf was cut into approximately 3 equal segments perpendicular to the main vein and placed adaxial side up on a sterile 3M (9-cm) Whatman filter paper soaked with 5ml sterile water to maintain humidity in an inverted Petri dish. Each leaf segment was inoculated on the right side of the main vein with 3 mm diameter agar plugs containing mycelium of *Phytophthora* obtained from the edge of a 5 day old colony. Two different species (*P. capsici* and *P. palmivora*) were used in separate experiments for inoculation of all lines. Control agar plugs without *Phytophthora* were placed on the left side of the leaf segments. Three *Phytophthora* and 3 control agar plugs were placed on each leaf segment.

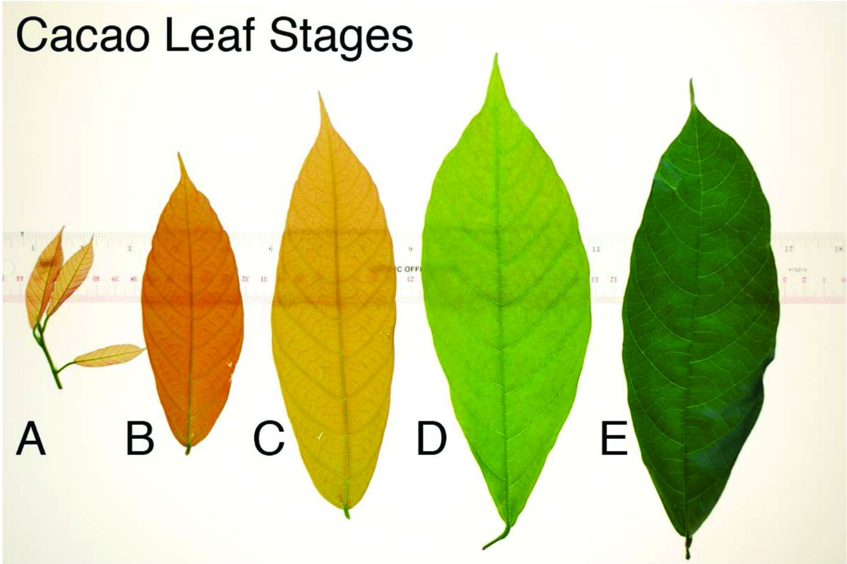


Figure 1. Developmental stages of Theobroma cacao (accession PSU-Sca6) leaf development imaged by trans-illumination. Stage A, emerging cacao leaves are translucent (note ruler visible through leaf) and thin, often with distinctive red color (some genotypes do not develop red pigments). Stage B and C, leaves undergo expansion but are still thin, translucent and pigmented. Stage D, elongation ceases and leaf accumulates chlorophyll, becomes light green, leaf is non-translucent and begins hardening. State E, leaf fully developed, leathery, and dark green. Ruler: major divisions on top - inches, bottom - centimeters.

The plates were incubated at 27°C in a 12:12h light:dark cycle under fluorescent light for 3 days, then imaged using a Nikon SMZ-4 dissecting and a 3 CCD video camera system (Optronics Engineering, Goleta, CA). The areas of necrosis was measured using ImageJ software (http://rsbweb.nih.gov/ij/) and averages of 24 replicates were calculated. The average area of necrosis developed from three inoculations per leaf and of five leaves per line were log transformed and compared using a one way ANOVA followed by a Tukey post hoc test.

Results

Direct Effect of Peptides D2A21, D5C, and D4E1 on *Phytophthora megakarya* Infection of Cacao Leaves

P. megakarya is the most recent, but the most destructive species of *Phytophthora* pathogens of cacao (*19*). Its occurrence is currently limited to cacao producing countries in Africa. The direct effect of peptides D2A21, D5C and D4E1 on spore germination of *P. megakarya* was evaluated. Experiments were performed by individually applying 20 µl of peptide solutions at different concentrations to detached cacao leaves from non-transgenic plants grown in the greenhouse. Peptide applications were followed by inoculations with zoospore suspension of *P. megakarya* (10^4/ml) overlaying the peptides on the leaves. Peptide concentrations tested ranged from 32 - 192 µM $^{\text{ml-1}}$ (Table III). The leaf discs were incubated at room temperature in Petri plates and the development of leaf necrosis caused by *P. megakarya* was recorded.

Results from these experiments demonstrated that at concentration of 64 µM $^{\text{ml-1}}$ all three peptides evaluated had the ability to stop the development of cacao leaf necrosis due to *P. megakarya* (Table III). Peptide D5C was the most effective against the pathogen and reduce necrosis development at a concentration of 32 µM $^{\text{ml-1}}$.

Generation of Transgenic Cacao Plants Expressing Antimicrobial Peptides

Secondary embryos regenerated from transformations with the individual constructs containing genes for antifungal peptides were screened by fluorescence microscopy for green fluorescence (expression of the co-linked *EGFP* marker). Five high-level EGFP expressing transgenic secondary somatic embryos were identified (Table I). The embryos were selected to represent individual transformation events. Cotyledon explants of the transgenic secondary embryos were introduced back into culture for regeneration of multiple tertiary embryos and the establishment of transgenic lines. Tertiary embryos from each line were converted into plantlets and acclimated to greenhouse conditions and further propagated by rooted cuttings (*20*). All plants evaluated in this study have been grown in a greenhouse since 2005. Phenotypic observations of the growth of the trees in the greenhouse indicated that there were no observable morphological differences between transgenic and non-transgenic somatic embryos-derived plants (Figure 2).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

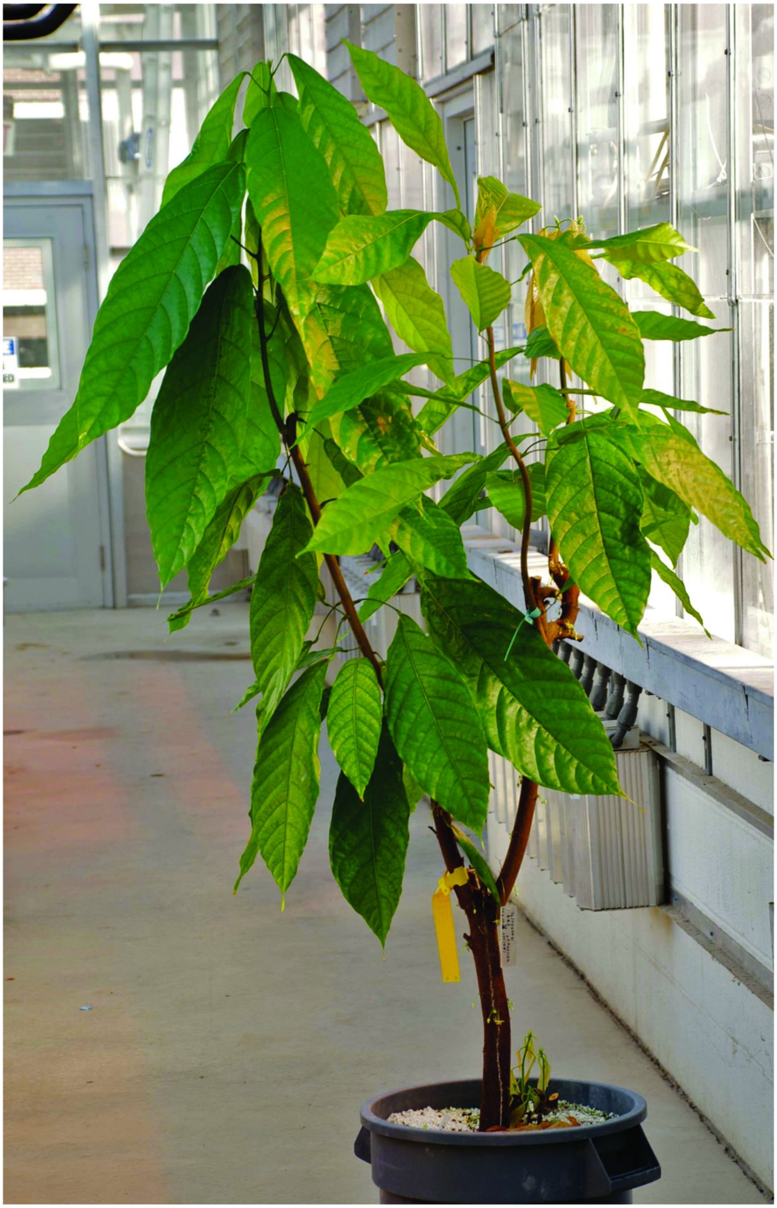


Figure 2. Theobroma cacao plant expressing antimicrobial peptide D4E1 (line 76). Plant shows normal development and phenotype indistinguishable from non-transformed control plants.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

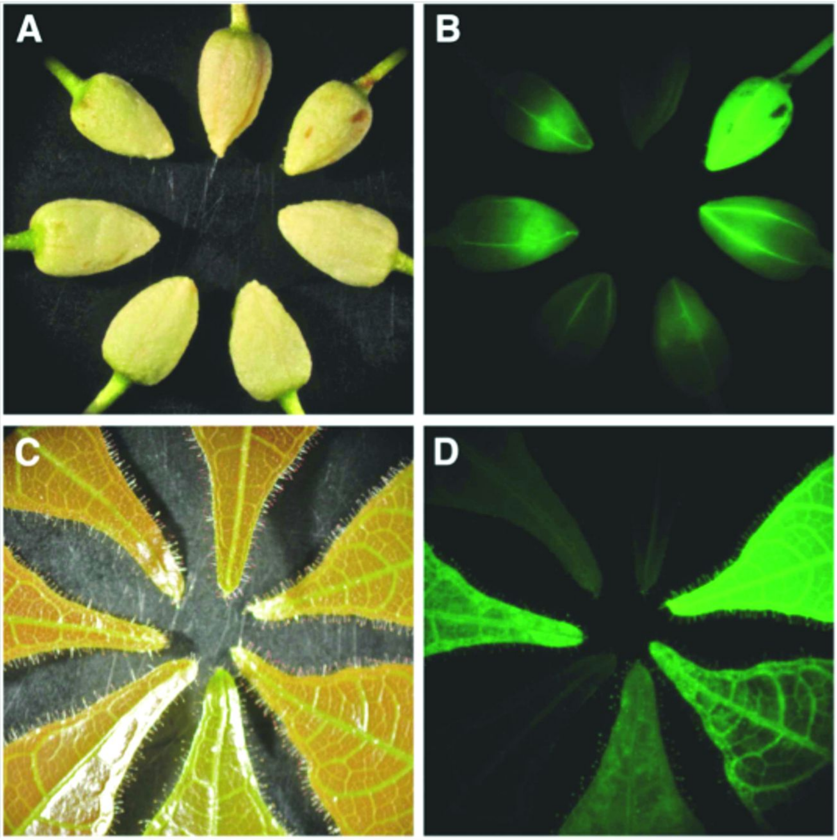


Figure 3. Photomicrographs of T. cacao plant tissues demonstrating expression of green fluorescent protein gene, which is adjacent to antimicrobial peptide genes. Tissues taken from mature plants (at least four years of age). (A) Light micrographs of immature flowers; (B) fluorescence image of same flowers depicted in A; (C) Light micrographs of young leaf tissues; (D) fluorescence images of same leaves as depicted in C. In each panel, plant genotypes are positioned in the same order clockwise direction from top-center: PSU-Sca6 (untransformed negative control), Transgenic Line 31 (control line, transformed with GFP gene without antimicrobial peptide genes), and Lines 70, 71, 74, 76 and 77, expressing anti-fungal peptides as described in the text.

GFP Fluorescence Intensity Variation in Transgenic Cacao

The intensity of green fluorescence in the flowers and mature leaves from the different transgenic lines was observed and compared to that of control leaves (Figure 3). The fluorescence intensity of individual leaf samples, varied significantly among the transgenic lines. This is similar to the our results obtained in a previous study were transgenic cacao lines were generated using vector

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

pGH0.0131 (*10*) and likely is a result of local effects of genome position on the expression of the GFP transgene. The images in Figure 3 were captured in 2011, demonstrating that the EGFP expression in the transgenic plants is stable after over 6 years of growth in the greenhouse. The transgenic constructs integrated into these plants contain the synthetic antimicrobial peptide genes immediately adjacent to the *EGFP* gene, and thus it is reasonable to assume that the peptide gene expression has also been maintained in the plants.

Table III. Effect of antimicrobial peptides on *Phytophthora megakarya*

Peptide	*Replicate*	*Concentration µM ml^{-1}*						
		0	*32*	*64*	*96*	*128*	*160*	*192*
D2A21	1	+	0	0	0	0	0	0
	2	+	+	0	0	0	0	0
	3	+	(+)	0	0	0	0	0
	4	+	+	0	0	0	0	0
D5C	1	+	0	0	0	0	0	0
	2	+	0	0	0	0	0	0
	3	++	0	0	0	0	0	0
	4	++	0	0	0	0	0	0
D4E1	1	++	+	0	0	0	0	0
	2	++	++	0	0	0	0	0
	3	+	++	+	0	0	0	0
	4	+	+	0	0	0	0	0

++ necrosis, + strong infiltration; (+) water soaking; 0 negative

Molecular Analysis of Transgene Insertion into the Genome and Gene Expression

The incorporation of the *EGFP* and the peptide genes *D5C-cyto* and *D4E1* was verified by genomic PCR (Figure 4). Plasmid DNAs containing the binary plasmids used in this study were diluted to genome equivalent amounts (15 pg of plasmid equals approx. molar-equivalent to 5 ng of cacao genomic DNA) using salmon sperm DNA as carrier (5 ng per reaction). No amplification was observed with all primer sets and the salmon sperm DNA (SS) used to dilute the plasmid DNAs (Figure 4, lane 2). The amplification of non-transgenic PSU-Sca6 (S6) with all primers resulted in only one fragment of 566 bp (spanning 96 bp intron sequence) corresponding to endogenous gene *TcActin* (Figure 4, lane 3). As expected, plasmid pGH00.0126 produced a 630 bp *EGFP* fragment (Figure

4, lane 4). Transgenic line 31 (without peptides) resulted in the amplification of *EGFP* and *TcActin* fragments (Figure 4, lane 5). Control plasmid pGM02.1023 produced two fragments corresponding to *EGFP* and *D5C-cyto* (86 bp) (Figure 4, lane 6). Genomic PCR of the 2 transgenic lines 70 and 74 transformed with pGM02.1023 generated 3 fragments corresponding to genes *EGFP*, *TcActin* and *D5C-cyto* (Fig. 4, lane 7 and 8). The control reaction with pGP02.1122 generated fragments corresponding to *EGFP* and a 1758 bp fragment corresponding to the region of the T-DNA including partial *EGFP* sequence, *35S-terminator* region from the *EGFP* cassette, the CaMV35S promoter region of gene *D4E1* and the coding sequence region of peptide gene *D4E1* (Figure 6, lane 9). Correspondingly the reaction including genomic DNA from transgenic line 76 generated with this plasmid produced the same fragment pattern as the plasmid also including the *TcActin* fragment. These results demonstrated that the *EGFP* and the peptide genes were stably incorporated into the genomes of these plants.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

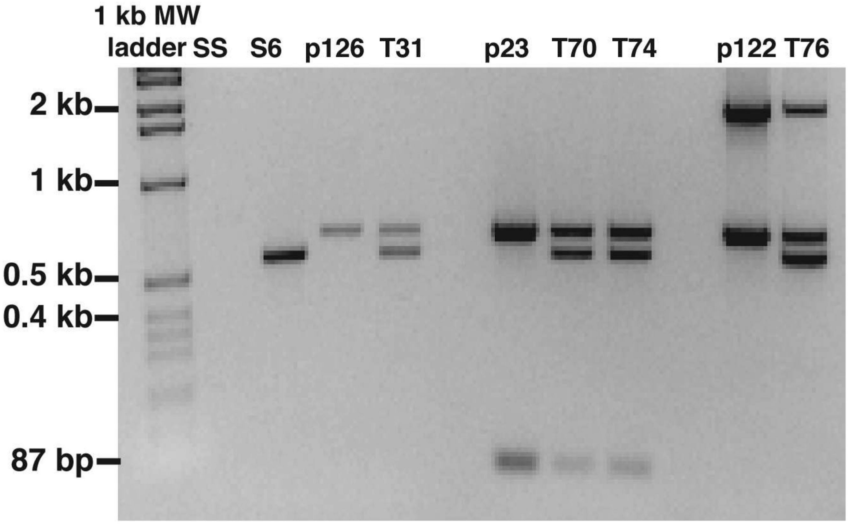


Figure 4. Genomic PCR analysis of non-transgenic and transgenic lines transformed with EGFP and peptide D5C-cyto or D4E1 genes. PCR amplification was performed with 4 primer sets: Cacao TcActin gene primers producing a 566 bp fragment, EGFP gene primers amplifying a 630 bp fragment, D5C-cyto primers amplifying a 87 bp fragment, and D4E1 primers amplifying a 1758 bp fragment. The control plasmid DNA samples used were from pGH00.0126 (p126) containing EGFP gene without peptide genes, pGM02.1023-D5C-cyto (p23), and pGP02.1122-D4E1 (p122). The plasmid DNA was diluted to 0.15 pg in salmon testes DNA (SS). Leaf DNA was extracted from non-transgenic PSU-Savina 6 (S6) and transgenic plants from 4 independent lines: Line 31 (control GFP, without peptides), lines 70 and 74 (transformed with pGM02.1023-D5C-cyto) and line 76 (transformed with pGP02.1122-D4E1).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

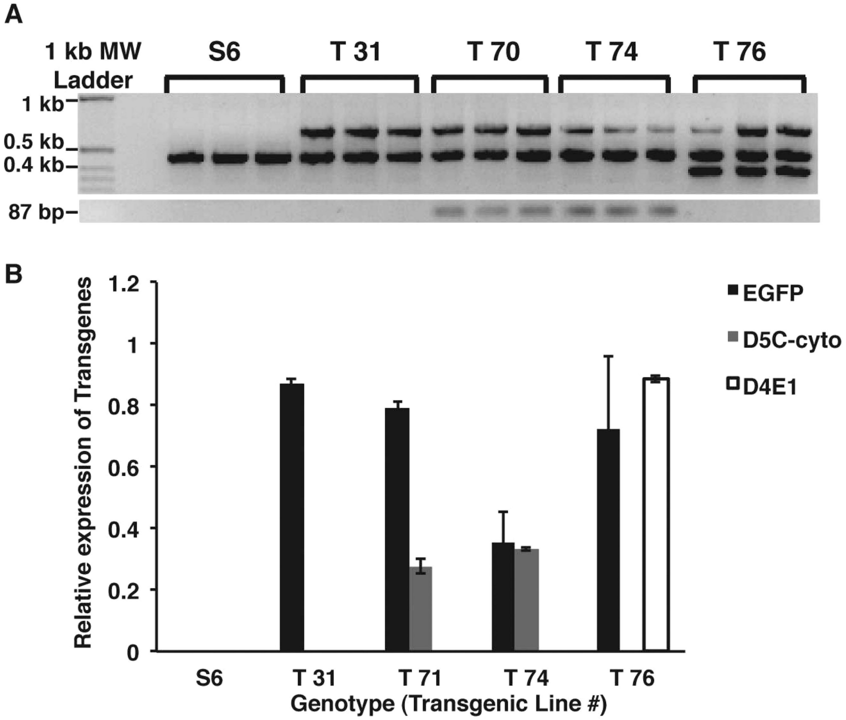


Figure 5. Transgene expression analysis in different genotypes of cacao. (A) Expression of TcActin, EGFP, D5C-cyto and D4E1 genes in cacao leaves. Three mature leaves were collected from each cacao genotype and RNA was extracted in 3 separate extractions. Semi-quantitative RT-PCR was performed. (B) Relative gene expression levels. Using ImageQuant software the color intensities of the fragments on the gel images were measured and relative transgene expression was quantified normalized to TcActin expression. Expression levels are presented as the means ± standard errors of three biological replicates.

Transgene expression was evaluated by semi-quantitative RT-PCR analysis using total RNA from mature leaved of non-transgenic and 4 transgenic lines (Figure 5). Compared to the non- transgenic PSU-Sca6 (S6), which resulted in amplification of only one 470 bp fragment corresponding to the control endogenous *TcActin* gene (Figure 5, lanes 3-5), the Line 31 (*EGFP* control transgenic) plant produced the *TcActin* gene fragment in addition to the *EGFP* fragment (630 bp). The analysis of transgenic RNA from lines 70 and 74 transformed with gene encoding for peptide D5C-cyto produced the two control fragments in addition to 87 bp fragment corresponding to length of the peptide gene. Similarly the amplification line 76 resulted in generation of the control fragments and a 366 bp fragment corresponding to the size of the *D4E1*gene plus the *Nopaline Synthase 3'-UTR*. This analysis verified the presence of the transcripts (gene expression) of the corresponding transgenes in the plants evaluated. To compare the relative expression of the individual transgenes within each sample the data were normalized to the expression level of an the endogenous actin gene expression of the same sample. The results indicated that *D5C-cyto* gene has significantly lower expression then *EGFP* in line 71, but the expression levels of *EGFP* and the same peptide in line 74 is very similar. The expression levels of *EGFP* and *D4E1* in line 76 are also similar to each other. This result are not surprising because the expression of all transgenes in this experiment is under the regulation of strong constitutive *CaMV35S* promoter.

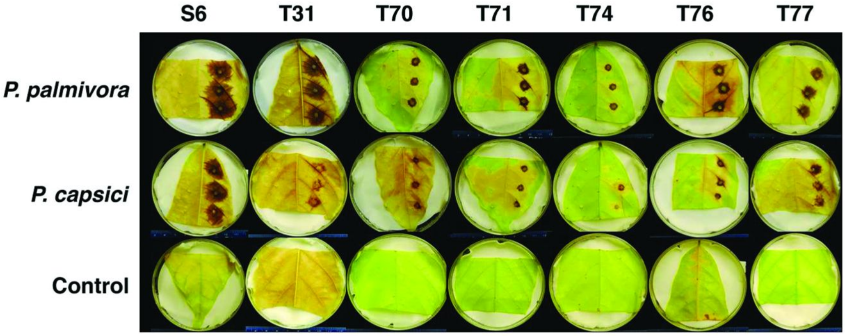


Figure 6. Representative images of necrosis caused by Phytophthora palmivora (top row) and Phytophthora capsici (middle row) observed on detached cacao leaves of control and transgenic T. cacao at 3 days post inoculation. Plant genotype (Line #) indicated on top. Each leaf was treated with agar plugs containing pathogen mycelium on the right side of each mid-vein and with agar plugs with no pathogen on the left side of each mid-vein. The bottom row depicts leaves which were not treated with agar plugs (controls).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

Evaluation of Disease Suppression in Transgenic Cacao Leaves Inoculated with *Phytophthora capsici* and *Phytophthora palmivora*

A preliminary experiment was conducted to optimize the *in vivo* detached leaf assay using 2 pathogenic species of *Phytophthora*. Infection and necrosis development caused by *P. capsici* strain 73-73 and *P. palmivora* strain 74-74 was assessed using detached leaves of two developmental stages of cacao genotype PSU-Sca6: leaf stages C and E (Figure 1). The assessment was performed using 3 mm diameter agar plugs with mycelium of *Phytophthora* obtained from the edge of a 5 days old colony. Reproducible necrotic lesions were obtained by placing agar plugs of both *P. capsici* and *P. palmivora* on the abaxial side of leaves of developmental stage C while no necrosis developed at stage E. Therefore further evaluation of the transgenic lines was performed on leaves at developmental stage C.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

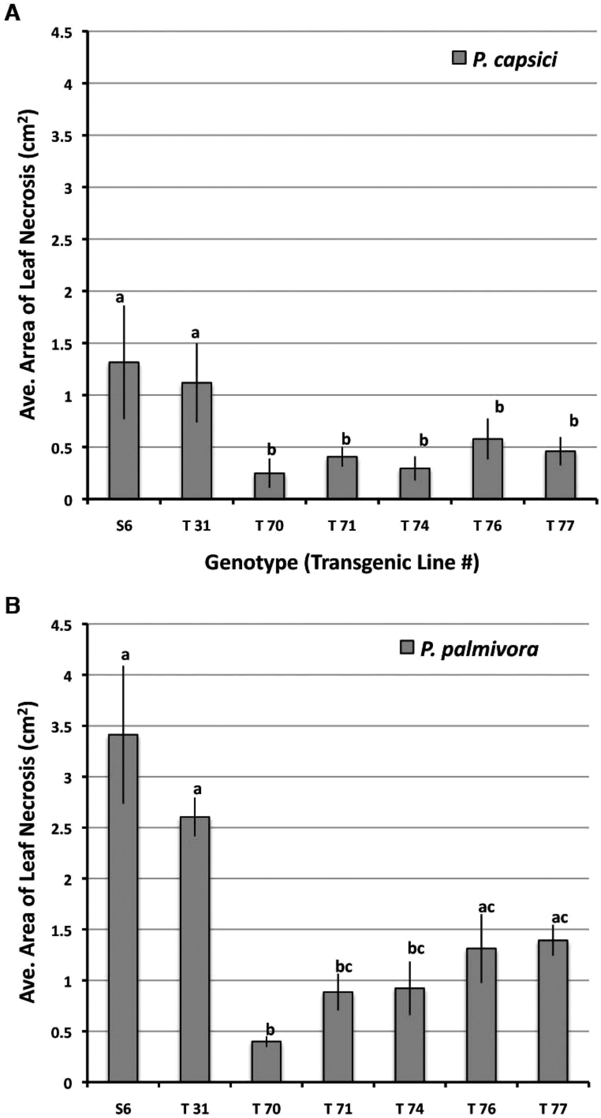


Figure 7. Necrotic lesion development on inoculated cacao leaves. Lesion sizes were measured from images such as depicted in Figure 4. Graphs represent mean areas of necrotic lesions ± SE on leaves at 3 days after inoculation with (A) P. capsici and (B) P. palmivora. Significant differences in average area of necrosis caused by Phytophthora were found across the different lines (ANOVA, F = 2.97 ; d.f. = 6, 28; P = 0.022 in experiment with P. capsici, F = 14.5; d.f. = 6, 28; P= 0.000 in experiment with P. palmivora).

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

To test the efficacy of the transgenic expression of antimicrobial peptides, leaves from each of transgenic and control plants were assayed for *Phytophthora* infection. Necrotic lesions with different sizes were observed in all genotypes inoculated with the individual pathogens after 3 days of incubation (Figure 6). No lesions were observed on leaves treated with agar plugs without pathogen or non-treated leaves. Lesion sizes were measured to determine the significant differences among the genotypes in response to the pathogen infection. The leaves from all peptide expressing transgenic lines inoculated with *P. capsici* demonstrated statistically significant decreases in lesion sizes compared to non-transformed control cacao leaves or with transgenic plants expressing EGFP only (no antimicrobial peptide expression), ($P = 0.000$, Figure 7). When assayed with *P. palmivora* the results were statistically significantly for only three of the lines as compared to the control plants (lines 70, 71, 74, $P = 0.022$, Figure 7).

Discussion

Our results demonstrate the efficacy of transgenic expression of antimicrobial peptides in cacao leaves against two major cacao pathogens. A useful property of antimicrobial peptides in agricultural applications is that a single peptide can have growth inhibitory activity against a broad range of microorganisms. Thus plants engineered for high expression of these peptides are potentially capable of antagonizing the growth of several microbial plant pathogens and controlling the development of the diseases they cause. For example the synthetic peptide D4E1 has been demonstarted to inhibit the growth of fungal, oomycetal and bacterial plant pathogens *in vitro* and *in planta* (*8*). It would be of interest to evaluate if a synergistic effect on pathogen growth would be observed in plants expressing multiple antimicrobial peptides or when combined with genes for other anti-pathogen genes such as the chitinase gene described above.

It is also important to consider that the useful broad range activity of antimicrobial peptides may have non-desirable effects, for example if they antagonize the growth of beneficial symbiotic microorganisms such as plant growth promoting rhizobacteria, endophytic fungi with biocontrol activity of pathogens, and arbuscular micorrhizal fungi. It is widely accepted that all plants harbor endophytic microorganisms that live in asymptomatic, frequently beneficial symbioses with them (*21*). Beneficial endophytic fungi usually belongs in genera that also contain plant pathogens therefore it is possible that antimicrobial peptides affecting pathogens will also affect closely related beneficial endophytic species. To our knowledge the potential effects of antimicrobial peptides on plant associated beneficial microorganisms have not been tested and studies on this regard should be done. Testing the effects of antimicrobial peptides on beneficial microorganisms may provide important knowledge as to the peptides mode and range of action and to how plant associated beneficial microorganisms overcome plant innate immune responses. It may be possible to target peptide accumulation to cellular domains where it is accessed only by pathogenic organisms but not symbiotic organisms.

Additionally assessment of the energetic cost for the plant to constitutively express antimicrobial peptides as was done in this work remains to be explored. It may be desirable to use promoter elements that express the peptide genes only during pathogen infection, which could reduce the energetic drain on the plant. The results obtained in this and other studies suggest that this technology has a good potential for limiting plant pathogen growth *in planta* thus can be helpful in controlling plant diseases, certainly the major limiting factor of cacao production.

Acknowledgments

We would like to thank Sharon Pishak, Ann Young, Enith Rojas and Sunshine Van Bael for assistance with data acquisition and analysis, and Demegen Inc. for providing the peptide constructs. This research was supported with funds from Penn State University and NSF/BREAD Award 0965353.

References

1. Cocoa Facts and Figures: World Cocoa Foundation, 2011. Available from URL: http://www.worldcocoafoundation.org/learn-about-cocoa/cocoa-facts-and-figures.html.
2. Ploetz, R. C. Cacao diseases: important threats to chocolate production worldwide. *Phytopathology* **2007**, *97* (12), 1634–9.
3. Maximova, S. N.; Marelli, J. P.; Young, A.; Pishak, S.; Verica, J. A.; Guiltinan, M. J. Over-expression of a cacao class I chitinase gene in *Theobroma cacao* L. enhances resistance against the pathogen, *Colletotrichum gloeosporioides*. *Planta* **2006**, *224*, 740–49.
4. Snyder T. *Isolation and characterization of a genomic chitinase clone form Theobroma cacao L.* Intercollege Program in Plant Physiology; The Pennsylvania State University: University Park, PA, 1994; p 46.
5. Marshal S.; Arenas G. Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology. *Electron. J. Biotechnol.* [serial online]. 2003. 6(3). Available from URL: http://www.ejbiotechnology.info/content/vol6/issue3/full/1/BIP/index.html.
6. Mygind, P. H.; Fischer, R. L.; Schnorr, K. M.; et al. Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. *Nature* **2005**, *437* (7061), 975–80.
7. Rajasekaran, K.; Stromberg, K. D.; Cary, J. W.; Cleveland, T. E. Broad-spectrum antimicrobial activity *in vitro* of the synthetic peptide D4E1. *J. Agric. Food Chem.* **2001**, *49* (6), 2799–803.
8. Cary, J. W.; Rajasekaran, K.; Jaynes, J. M.; Cleveland, T. E. Transgenic expression of a gene encoding a synthetic antimicrobial peptide results in inhibition of fungal growth *in vitro* and *in planta*. *Plant. Sci.* **2000**, *154* (2), 171–81.
9. Maximova, S; Miller, C.; Antunez de Mayolo, G.; Pishak, S.; Young, A.; Guiltinan, M. J. Stable transformation of *Theobroma cacao* L. and influence

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

of matrix attachment regions on GFP expression. *Plant Cell Rep.* **2003**, *21* (9), 872–83.

10. De Block, M.; Herrerra-Estrella, L.; Van Montagu, M.; Shell, J.; Zambryski, P. Expression of foreign genes in regenerated plants and their progeny. *EMBO J.* **1984**, *3*, 1681–89.
11. Mitsuhara, I.; Ugaki, M.; Hirochika, H.; et al. Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol.* **1996**, *37* (1), 49–59.
12. Lazo, G. R.; Stein, P. A.; Ludwig, R. A. A DNA Transformation-Competent Arabidopsis Genomic Library in *Agrobacterium*. *Biotechnology* **1991**, *9*, 963–67.
13. Lin, J. Optimization of the transformation efficiency of *Agrobacterium tumefaciens* cells using electroporation. *Plant Sci.* **1994**, *101*, 11–15.
14. Antúnez de Mayolo, G.; Maximova, S. N.; Pishak, S.; Guiltinan, M. J. Moxalactam as a counter-selection antibiotic for *Agrobacterium*-mediated transformation and its positive effects on *Theobroma cacao* somatic embryogenesis. *Plant Sci.* **2003**, *164*, 607–15.
15. Li, Z.; Traore, A.; Maximova, S.; Guiltinan, M. J. Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao L.*) using thidiazuron. *In Vitro Cell. Dev. Biol.: Plant.* **1998**, *34*, 293–99.
16. Maximova, S. N.; Alemanno, L.; Young, A.; Ferriere, N.; Traore, A.; Guiltinan, M. Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. *In Vitro Cell. Dev. Biol.: Plant.* **2002**, *38* (May-June), 252–59.
17. Verica, J. A.; Maximova, S. N.; Strem, M. D.; Carlson, J. E.; Bailey, B. A.; Guiltinan, M. J. Isolation of ESTs from cacao (*Theobroma cacao* L.) leaves treated with inducers of the defense response. *Plant Cell Rep.* **2004**, *23* (6), 404–13.
18. Brechenmacher, L; Weidmann, S.; van Tuinen, D.; et al. Expression profiling of up-regulated plant and fungal genes in early and late stages of *Medicago truncatula*-Glomus mosseae interactions. *Mycorrhiza* **2004**, *14* (4), 253–62.
19. Hebbar, P. K. Cacao diseases: a global perspective from an industry point of view. *Phytopathology* **2007**, *97* (12), 1658–63.
20. Maximova, S. N.; Young, A.; Pishak, S.; Miller, C.; Traore, A.; Guiltinan, M. J. Integrated system for propagation of *Theobroma cacao* L. In *Protocol for somatic embryogenesis in woody plants*; Jain, S. M., Gupta, P. K., Ed.; Springer: Dordrecht, The Netherlands, 2005; pp 209−29.
21. Rodriguez, R. J.; White, J. F., Jr.; Arnold, A. E.; Redman, R. S. Fungal endophytes: diversity and functional roles. *New Phytol.* **2009**, *182* (2), 314–30.

Downloaded by UNIV OF GUELPH LIBRARY on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch018

Chapter 19

Antimicrobial Peptides for Fire Blight Control

V. Sarojini*

School of Chemical Sciences, The University of Auckland,
23 Symonds Street, Auckland Central, Private Bag 92019,
Auckland 1142, New Zealand
***E-mail: v.sarojini@auckland.ac.nz**

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Fire blight, caused by the Gram negative bacterium *Erwinia amylovora*, is an economically devastating disease of pome fruit trees affecting North America, Europe, New Zealand and several Eastern Mediterranean countries (*1*, *2*). Currently used chemical control options, except streptomycin, do not provide satisfactory results in serious outbreaks. The development and spread of streptomycin resistant strains of *E. amylovora* in countries like New Zealand, North America and Israel make it necessary to develop novel compounds, possibly having different biological targets, to control this disease. Antimicrobial peptides are attractive candidates as alternatives to conventional antibiotics, since they have less chance of resistance development and exhibit novel mechanism of action (*3*). Research from various laboratories around the world during the past two decades has identified several naturally occurring antimicrobial peptides and their synthetic analogues with potential for fire blight control. Even though these molecules are yet to find entry into the commercial sector for chemical control of fire blight, there is ample evidence of their potential to provide the necessary and desired alternative to antibiotics for fire blight control.

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Introduction

Antibiotic resistance is a growing public health problem the world over. The antibiotics that saved millions of lives since the discovery of Penicillin in the 19th century are now at risk, as high levels of drug resistance threaten their effectiveness. These multidrug resistant (MDR) microorganisms cling together to surfaces (biotic or abiotic) and use extracellular polymer matrices as a barrier to drug penetration. They are commonly known as biofilms. Biofilm formation by human, animal and plant pathogens lead to high health costs to humans and severe economic loss in the agricultural sector. *Pseudomonas aeruginosa* that inhabit the lung tissues of cystic fibrosis patients and *Erwinia amylovora* the causative agent of fire blight, are examples of bacterial biofilms that cause major concerns in different fields (*4*, *5*). Antimicrobial peptides (AMPs) that form part of innate immunity in almost all forms of life are attractive candidates for the development of novel treatment options to tackle the multi drug resistant bacterial biofilms (*6–8*). This chapter will highlight the research from various laboratories on peptide based molecules that have shown antibacterial activity against *E. amylovora* and are hence promising candidates for fire blight control.

Fire Blight, Its Economic Importance, and Its Causative Agent

Fire blight affects the entire plant causing five distinct types of infection known as blossom blight, shoot blight, canker blight, fruit blight and rootstock blight (*9*, *10*). Severe outbreaks of fire blight necessitate the removal of whole orchards resulting in significant economic loss for fruit growers. Millions of dollars worth of damages have been reported from affected countries, especially U.S.A. and New Zealand (*11*, *12*). Prevalence of fire blight in New Zealand has restricted market accessibility of New Zealand grown apples putting additional economic burden on farmers (*13*). Given the existing scenario with the rapid spread of fire blight into newer apple and pear growing regions, the economic impact caused by this disease is only going to continue to increase.

E. amylovora, the causative agent of fire blight, is a Gram negative bacterium belonging to the *Enterobacteriaceae* family (*10*). Numerous factors are known to contribute to the pathogenicity of *E. amylovora*. These include the production of the exopolysaccharides, amylovoran and levan, the harpin proteins, the siderophore desferroxamine as well as the quorum sensing signalling molecules (*10*, *14*, *15*). More recently it has been shown that *E. amylovora* forms bioflms and that biofilm formation is crucial for the pathogenicity of this bacterium (*4*).

Fire Blight Management

The options available for managing fire blight are limited as well as costly. The common methods available to farmers for minimizing the spread of the disease like removal of the source of infection, spraying chemicals to protect the plant against the pathogen, avoidance of susceptible cultivars etc. do not provide an effective management strategy.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Existing Chemical Control Options and Their Drawbacks

Spray applications of streptomycin and copper compounds are the two most widely used chemical control options registered for fire blight in most countries affected by this disease (*16*). However, these options have significant drawbacks of resistance development and phytotoxicity respectively. The use of streptomycin, for fire blight control, is becoming more and more limited because of the spread of resistant strains of the bacterium as well as the existence of regulatory measures in many countries in the EU where the use of clinically relevant antibiotics in horticulture is prohibited (*17*). Certain countries in Europe use other chemicals like Flumequin, Fosetyl-Al and oxolinicacid for fire blight control. None of these have entered the global scenario yet. Table 1 lists the chemical control options used for fire blight control in different countries.

Table 1. Chemicals registered for use against fire blight in different countries

Compound	*Trade Name*	*Country*	*Comments*
Flumequin	Firestop™ Fructil™ MBR10995	France, Belgium and Cyprus	Not approved in NZ, U.S.A. and Canada
Fosetyl-Aluminum (Fosetyl-Al)	Aliette™	France and Turkey	Inconsistent results from year to year and trial to trial
Oxolinic acid	Starner™	Italy	Mostly used for preventive treatment
Copper Compounds	Available under various trade names	NZ, US, Netherlands, Italy,Canada, Belgium, Greece (under several different trade names)	Phytotoxicity
Streptomycin	AgrimycinR17, Plantomycinetc	NZ, US, Netherlands, Italy, Canada, Belgium, Greece (under several different trade names)	Resistant strains identified in U.S.A., Canada, New Zealand and Israel

Copper Compounds

Bordeaux mixture containing a mixture of copper sulphate ($CuSO_4$) and lime [$Ca(OH)_2$] is a bactericide used for fire blight control since early 1900s (*18*). However, phytotoxicity is a major drawback of copper compounds (*16*, *19*). Additionally, the fact that several other phytopathogenic bacteria have become resistant to copper raises the concern of a similar resistance developing in *E. amylovora*, further restrictng the use of copper as a desirable fire blight control in future (*20*, *21*).

Antibiotics

According to the original definition by Selman Waksman, 'an antibiotic is a chemical substance produced by a microorganism that has the capacity to inhibit the growth and even destroy bacteria and other microorganisms' (*22*). The restricted use of copper compounds because of their phytotoxicity resulted in the use of antibiotics in horticulture immediately after the wonder drug penicillin saved thousands of human lives during the time of World War II in the 1940s (*23*)–(*25*). Several antibiotics, such as penicillin, neomycin, erythromycin, chloramphenicol, chloromycetin, tetracycline, streptomycin etc. were tried for their ability to control *E. amylovora* infections *in vitro* as well as in the fields (*26*, *27*). Amongst these, only streptomycin, oxytetracycline and kasugamycin satisfied the requirements for use in field trials (*16*). However, the use of kasugamycin is restricted because of its phytotoxicity (*28*, *29*). Oxytetracycline is inferior to streptomycin in fire blight control and hence has been used in combination with streptomycin especially to minimise the development of streptomycin resistant strains of *E. amylovora* (*30*).

The Growing Problem of Antibiotic Resistance: Antimicrobial Peptides as a Potential Solution

Antibiotic resistance is constantly on the rise threatening the effectiveness of several life-saving durgs. In order to raise awareness about this ever increasing problem, The World Health Organization chose 'combat antimicrobial resistance' as the theme for the World Health day 2011 and has prompted the scientific community to develolp novel compounds to treat multi drug resistant microorganisms. Antimicrobial peptides are desired alternatives to conventional antibiotics for the prevention and treatment of various microbial infections (*3*, *8*).

Antimicrobial peptides (AMPs) are produced by various organisms including plants, insects, microbes, amphibians and fish in order to battle against invading pathogens. AMPs are cationic in nature and contain 15 to 45 amino acids in their sequences (*31*, *32*). Their secondary structures vary from amphipathic helices, two to four stranded β-sheets, extended structures and loops. Naturally occurring AMPs show broad spectrum activity against several microorganisms, exhibit drug synergism with other antibiotics and have very few side effects (*3*, *33*).

Despite the major advantages peptides have over conventional antibiotics, peptide research had a relatively slow growth until the Nobel prize winning work

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

by du Vigneaud on the synthesis of the peptide hormones oxytocin and vasopressin (*34*). Initial advances in protecting group chemistry through the contributions of Bergmann and Zervas, Bodanzky, Carpino and others followed by the Solid Phase Peptide Synthesis pioneered by Robert Bruce Merrifield made the chemical synthesis of relatively large polypeptides amenable (*35–42*). Currently, peptide drugs sold worldwide account for more than 12 billion dollars (*43*). Several AMPs are under various stages of clinical trial developments (*44–48*). However, the low bioavailability of peptides is still considered as a drawback in their marketability as pharmaceuticals, despite the fact that the peptide drugs insulin and erythropoietin are still available only as injections (*33*). The advances in the chemical synthesis of peptides make it possible to develop novel synthetic peptides with enhanced bioavailability incorporating the desirable properties of naturally occurring AMPs to treat various microbial infections.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Peptides for Fire Blight Control

Protein/peptide based molecules reported in the literature for fire blight control belong to three categories – 1) large proteinaceous toxins like bacteriocins, 2) AMP genes expressed in transgenic plants to enhance resistance to fire blight and 3) smaller AMPs and their synthetic variations. The best example in the first category is Serracin P, a high molecular weight (HMW) bacteriocin isolated from *Serratia plymithicum* J7 culture supernatant that showed antibacterial activity against 24 pathogenic strains of *E. amylovora* from different countries (*49*). Examples of AMP genes expressed in plants include the work from Norelli's group where the expression of the lytic protein gene attacin E in orchard grown apple trees has been shown to confer increased resistance to fire blight (*50*, *51*). Another example in this category is the enhanced resistance to *E. amylovora* exhibited by Royal Gala apple shoots transformed by the MB39 gene which is a modification of the lytic protein cecropin SB37 (*52*, *53*). This review will elaborate on the third category listed above – namely small antimicrobial peptides and their synthetic variations that have shown potential for fire blight control either in the laboratory or field trials or both. The main focus will be on peptide based molecules with potential for fire blight control reported from New Zealand. This will be followed by a brief mention of other peptide antibiotics produced by strains of *Pantoea agglomerans* and finally synthetic analogues of magainin, and the cecropin-mellitin hybrid peptides that have shown potential for fire blight control.

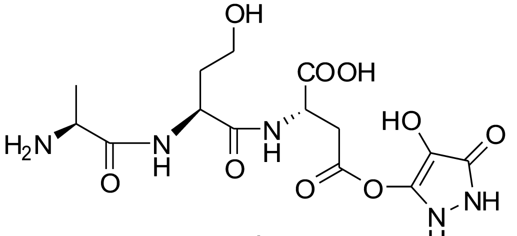
Antimicrobial Peptides for Fire Blight Control Reported from New Zealand

Antimicrobial compounds that suppress the growth of the fire blight pathogen *Erwinia amylovora* have been discovered from a wide range of *Pseudomonas* and *Pantoea agglomerans* strains (syn. *Erwinina herbicola*) in New Zealand and elsewhere (*54–64*). These antimicrobial compounds, on their own or through synthetic manipulations, provide useful means for potential control of fire blight.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Pyrazole-Conjugated Peptides

Mitchell has done extensive screening of numerous strains of the *Pseudomonas* species in an effort to find novel chemical control options for fire blight. Tropolone and a peptide derivative isolated from liquid cultures of *Pseudomonas* species were found to inhibit *E. amylovora in vitro* and on immature pear fruits (*57*, *58*). However, the level of infection on immature pear fruits treated with tropolone was raised to 100% after five days, but the tripeptide derivative provided complete protection from infection after five days. More recent work from Mitchell's group which included extensive ^{1}H and ^{13}C nuclear magnetic resonace (NMR) and mass spectral (MS) investigations on the peptide derivative confirmed it to be L-alanyl-L-homoserinyl-L-aspartic acid with an unusually substituted pyrazole ring linked to the beta carboxylic acid group of the C-terminal aspartic acid, **1** (*65*).



1

The unusually substituted oxopyrazole ring was found to be critical for the observed antibacterial activity of this compound. The antibacterial activity of **1** was suppressed by L-glutamine, indicating glutamine synthesis pathway as the potential target of this peptide. The authors reported that several other bacterial species were susceptible to the oxopyrazole peptide and thus this pyrazole conjugated peptide had a much broader spectrum of antibacterial activity. Table 2 summarises the antibacterial activity reported for this peptide.

The technical advances in computers and information technololgy during the past two decades has made computer-aided drug design an integral part of the modern drug discovery process. Today, the discovery and development of novel drugs, identification of novel biological targets for existing pathological conditions are all done with the aid of advanced computer technology (*66*). A combination of *in vitro* screening, synthetic chemistry and advanced spectroscopic techniques has led to the design, structure elucidation and discovery of novel peptides, in some cases with biological activities (*65*, *67*, *68*). We have recently extended our tool box to the use of advanced molecular modeling software to aid in the design of novel antimicrobial peptides. This approach was used to identify other potential targets for the oxopyrazole peptide as described below.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Table 2. Antibacterial activity of the oxopyrazole peptide (*65*)

Name of the bacterium	*Activity of the oxopyrazole peptide**
Erwinia amylovora	+
*Pectobacterium carotovorum (*synonym: *Erwinia carotovora)*	+
*Pectobacterium atrosepticum (*synonym: *Erwinia carotovora* pv. *atroseptica)*	+
*Pectobacterium carotovorum subsp. carotovorum (*synonym: *Erwinia carotovora* pv. *carotovora)*	+
Pseudomonas syringae pv. *syringae*	+
Pseudomonas syringae pv. *tomato*	+
Xanthmonas capmpestris pv. *pruni*	+
Pseudomonas corrugata	-
Pseudomonas marginalis	-

+ = active; - = not active

Similarity of the oxopyrazole ring in **1** to the nucleoside antibiotic pyrazofurin, isolated from *Streptomyces candidus*, prompted De Zoysa *et al* to undertake molecular modelling and docking experiments to evaluate the possibility that these two molecules could have similar biological targets in the bacterium (*69*). Pyrazofurin inhibits Orotatephosphoribosyltransferase (OPRT) and orotidine-5-phosphate decarboxylase (ODC) that catalyze the final steps in the biosynthesis of uridine-monophosphate (UMP) the building block for DNA synthesis (*70*). The molecular modelling studies, by De Zoysa *et al.*, showed that the oxopyrazole peptide completely overlaps the natural ligand (orotic acid) and binds more strongly to the active site of OPRT (*69*). Figure 1 shows the oxopyrazole peptide and orotic acid docked separately onto the binding site of the enzyme OPRT. The additional hydrogen bonding interactions of the peptide with the OPRT binding site that involve the side chains of residues Lys (*27*) and Lys (*76*) are circled.

The oxopyrazole ring carrying different substituents were synthesized and tested for activity, on their own or coupled to form peptide linkages, against *E. amylovora*. Figure 2 shows the various synthetic analogues of **1** (**2**-**6**) investigated for *in vitro* activity against *E. amylovora*. (De Zoysa et al., manuscript). Only the brominated oxypyrazole and its peptide analogues inhibited the growth of *E. amylovora*. However, the antibacterial activity of these analogues was not as prominent as the natural product which further confirms that changes to the substitution pattern of the oxopyrazole ring can be detrimental for antibacterial activity. Further work on the modelling, synthesis and activity studies on these peptides is currently in progress in the author's laboratory.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

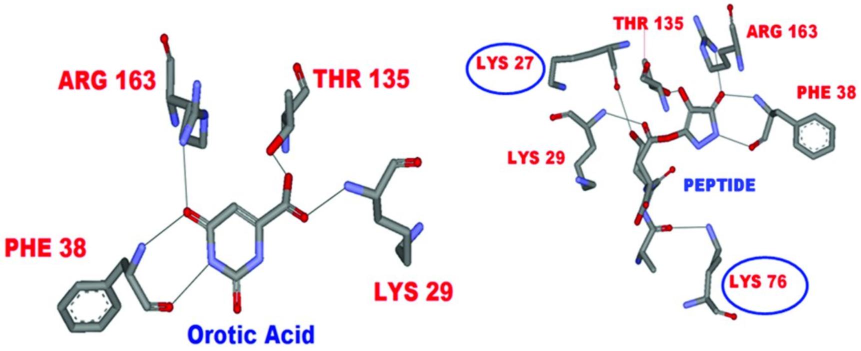


Figure 1. Hydrogen bonding interactions of co-crystalisedorotic acid (natural ligand of OPRT) and the docked oxopyrazole peptide with the binding pocket of OPRT.

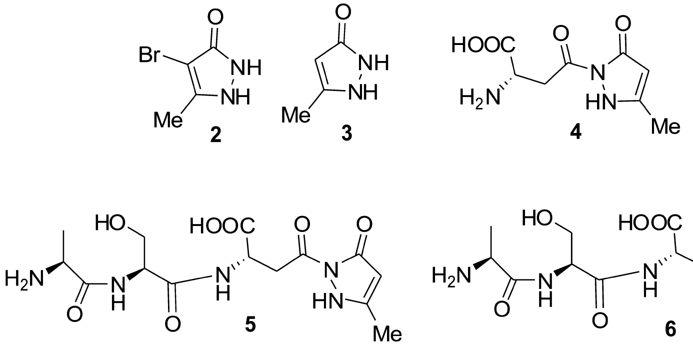


Figure 2. Synthetic analogues of the oxopyrazole ring and its peptide conjugates.

Iminopyrrolidine- and Piperidine-Containing Peptides

Two related compounds, with strong *in vitro* inhibitory activity against *E. amylovora*, were isolated by Mitchell and Teh from liquid cultures of *Burkholderia plantarii* (syn. *Pseudomonas plantari*) in 2005 (*71*). Extensive NMR and MS studies established the bioactive compounds to be 2-imino-3-methylene-5-L(carboxy-L-valyl)-pyrrolidine **7** and 2-imino-3-methylene-5-L(carboxy-L-threonyl)-pyrrolidine **8**. Both compounds, at 1 µg, resulted in clear inhibitory zones of approximately 20 mm diameter on agar plates overlaid with mid-log phase cultures of *E. amylovora*. The bioactivity of these compounds was found to be closely linked with the conjugated imino alkene functionality. All four stereomers of **7** were synthesised and tested for activity against *E. amylovora*, which confirmed that the biologically active natural product has the L,L stereochemistry (*72*). The 2-imino-3-methylene-pyrrolidine structure was also coupled to several hydrophobic amino acids, the synthetic peptides

(**9-16**) purified to homogeneity, structurally characterized and tested for activity against *E. amylovora*. All compounds showed strong *in vitro* activity against *E. amylovora*, even though compounds **9**, **11** and **16** were 2.5, 5 and 20 fold less active than the natural products. This study also included the syntheses of the higher homologs – the six membered ring piperidines coupled to L-Val, L-Ile and L-Thr (**17-19**) all of which showed strong *in vitro* inhibitory activity, comparable to the natural products **7** and **8**, against *E. amylovora* (*72*). The structures of the natural product and synthetic peptides containing iminopyrrolidine and piperidine that showed *in vitro* activity against *E. amylovora* reported by Mitchell are shown in Figure 3.

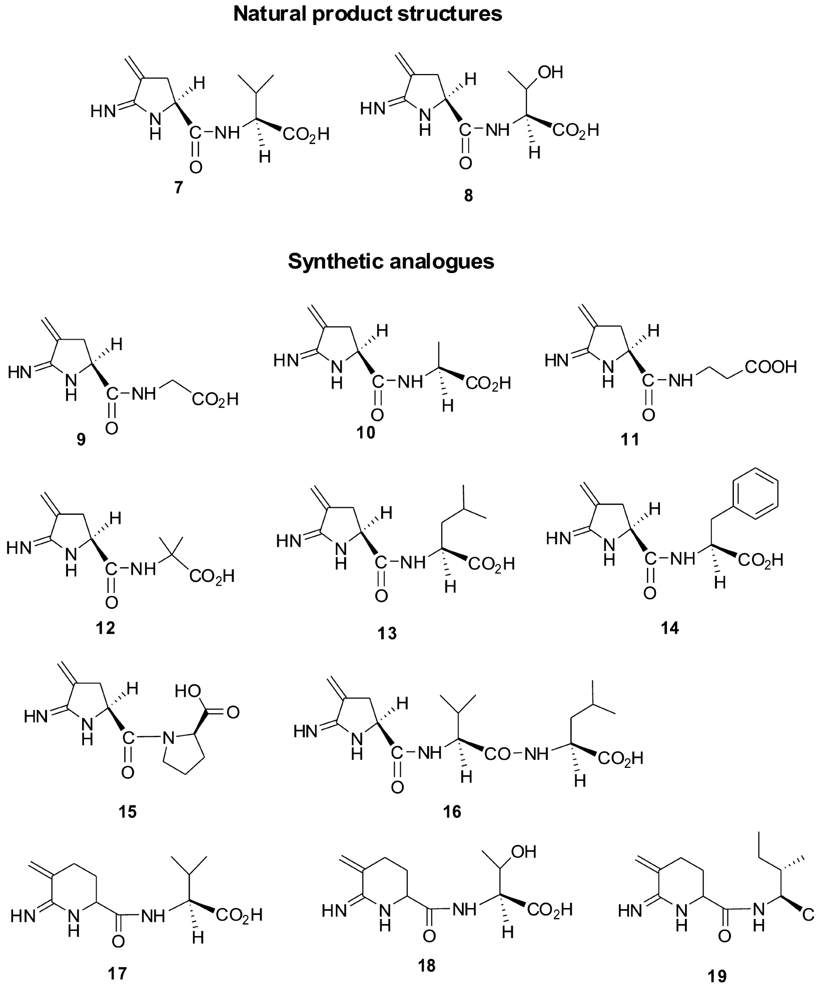


Figure 3. Iminopyrrolidine and piperidine containing peptides with activity against E. amylovora (72).

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Inhibiting Growth and Biofilm Formation in *E. amylovora*

De Zoysa *et al.* reported two non-natural amino acids with strong *in vitro* activity and biofilm inhibitory properties against *E. amylovora* (*73*). Both compounds were more potent than streptomycin against *E. amylovora* strain 1501. The compounds also had strong *in vitro* activity against the streptomycin resistant strain (Str4 Ea) of *E. amylovora*. Figure 4 shows the *in vitro* antibacterial activity of the lead compound, referred hereto as **A,** against Ea 1501 and Str4 Ea strains of this bacterium detected as zones of no bacterial growth surrounding the point of application of the compounds on agar plates overlaid with mid-log phase cultures of the respective bacterial strains. EA medium which consisted of K_2HPO_4 11.5 g, KH_2PO_4 4.5 g, $MgSO_4$ 0.2 g per 1 litre and L-asparagine 0.3 g, L-nicotinic acid 0.05 g and D-glucose 20 g was used for growing *E. amylovora* for these experiments.

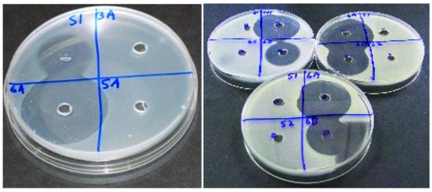
The top left quadrant in figure 4 (a) corresponds to the anti bacterial activity of 10 µg of streptomycin and the bottom left quadrant corresponds to that of 10 µg of inhibitor **A** against *E. amylovora* 1501. The larger and smaller inhibitory zones in figure 4 (b) correspond to 10 and 1 µg respectively of inhibitor **A** against the streptomycin resistant strain of *E. amylovora*. Streptomycin was used as the control in these experiments. Since plate (b) was overlaid with the resistant strain of *E. amylovora*, as expected, no inhibitory zone was detected surrounding the point of application of streptomycin on this plate. The diameters of the zones of inhibition measured from these plates are summarised in table 3. The MIC of A towards both bacterial strains was below 18 µM.

Inhibitor **A** was not phytotoxic to apple flowers or tobacco leaves at concentrations 40 times higher than the MIC value (De Zoysa et al, manuscript under preparation). An immature pear fruit assay (*74*) was used to evaluate the ability of the inhibitor to reduce the incidence of fire blight under a controlled environment. Several other potentially inhibitory compounds to the fire blight pathogen were also included in this experiment. Results obtained from this assay, plotted as the number of fruits infected under the different assay conditions, are shown in figure 5. As can be seen from this figure, the efficacy of inhibitor **A**, to prevent infection on immature pear fruits, is equal to streptomycin when $10^2$to 10^3cfu/ml of *E. amylovora* was used (De Zoysa et al, manuscript under preparation). We are currently evaluating the ability of the inhibitor to control infection in immature pear fruits at higher *E. amylovora* concentrations. The results also indicate that the other compounds tested in this experiment are less efficacious than streptomycin for fire blight control.

Our research group is also involved in the development of antimicrobial peptides for tackling biofilm forming human pathogens like *Pseudomonas aeruginosa* and *Staphylococcus aureus*. We extended our biofilm protocols to test the ability of inhibitor **A** to interfere with biofilm formation in streptomycin sensitive and resistant strains of *E.amylovora*. Representative microscopic images of *E. amylovora* 1501 biofilms stained with crystal violet in the absence (figure 6 a) and presence (figure 6 b) of the inhibitor are shown in figure 6. Comparison of these two images clearly indicates that inhibitor **A** prevents biofilm formation in Ea 1501. Similar results were obtained for the streptomycin resistant strain of

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

E. amylovora as well.Several replicates were used in these experiments and the results were also validated by different staining techniques.



(a) **(b)**

*Figure 4. Antibacterial activity of inhibitor **A** in comparison to streptomycin against (**a**) the streptomycin sensitive strain Ea 1501 and (**b**) the streptomycin resistant strain Str4 Ea.*

Table 3. Diameter of the zones of inhibition of Ea 1501 and Str4 Ea

Bacterial strains used	*Diameter of inhibitory zones in cm*			
	Streptomycin		*Inhibitor **A***	
	10 μg	*1 μg*	*10 μg*	*1 μg*
Ea 1501	2.7	1.7	4.3	3.0
Str4 Ea	0	0	4.2	3.0

These results indicate that the compound by itself or its synthetic variations has the potential to be developed as novel chemical control options for fire blight. The nature of the antibacterial activity of this inhibitor was investigated by co-inoculating different protein amino acids with **A** in the bioassays. Results showed that L-proline at a concentration of 100 mM prevents the antibacterial activity of **A**. Several peptides incorporating this inhibitor were synthesized and tested for activity against *E. amylovora*. All, except the proline containing peptide, showed strong activity against the bacterium, which is again indicative of the proline biosynthesis pathway as a potential target. This work is currently in progress in the author's laboratory.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

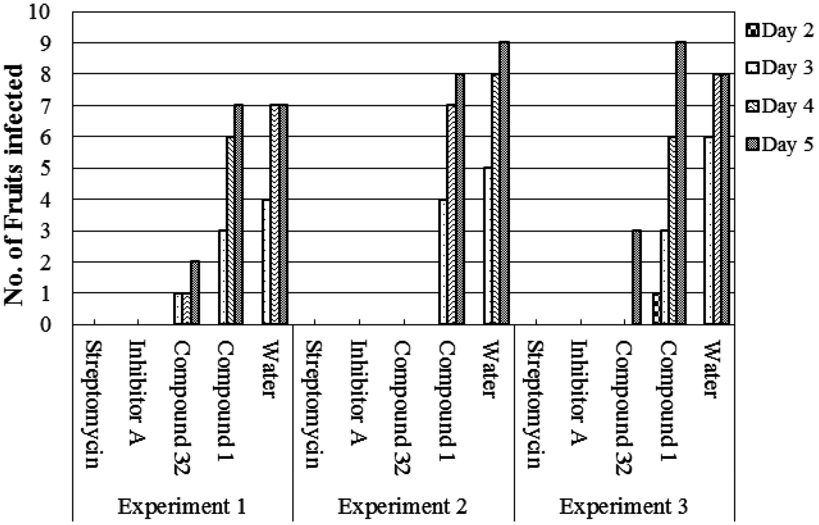


Figure 5. Number of cores of immature pear fruits showing signs of fire blight infection after treatment with different compounds and inoculation with E. amylovora [6.7 × 10^2 (experiment 1), 1.1 × 10^3 (experiment 2) and 6.3 × 10^2(experiment 3) cfu/ml].

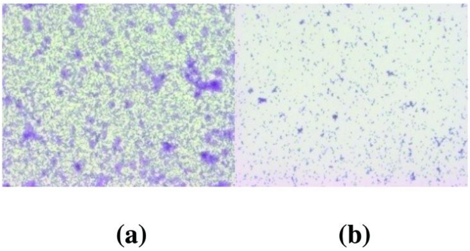
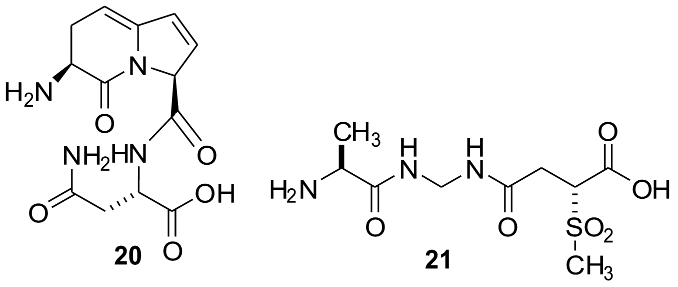


*Figure 6. Representative microscopic images of E.amylovora1501 biofilms stained with crystal violet (400× magnification) (**a**) control (**b**) in presence of inhibitor **A**.*

Antimicrobial Peptides Produced by *Pantoea*

*Pantoea vagans (*syn. *P. agglomerans, Erwinia herbicola)* is a non-pathogenic bacterium closely related to *Erwinia amylovora* that colonises the same plant surfaces as *E. amylovora*. As mentioned above, there are numerous literature reports on the production of antibiotics by *P. agglomerans*, some of which have been shown to have activity against *E. amylovora* (*55*, *56*, *60–63*). Herbicolin O produced by *Erwinia herbicola* strain C9-1 is a broad spectrum antibiotic that inhibits the growth of several Gram negative bacterial species including the fire blight pathogen (*56*). The chemical structure of Herbicolin O is not yet published. However, its similar molecular weight to Pantocin A and the fact that both compounds lose their antibacterial activity in the presence of L-histidine has led some researchers to speculate that Herbicolin O could be the same as Pantocin A, produced by *P. agglomerans* strain Eh318. Eh318 has been shown to produce another antibiotic, named Pantocin B with strong inhibitory activity against *E. amylovora* (*60*, *62*). The structures of Pantocin A and B are shown in figure 7. Pantocin B inhibits arginine biosynthesis in the bacterium. Both have a peptidic nature to their structures. The authors reported that, in minimal media, Pantocin B has picomolar activity against *E. amylovora*.



20 **21**

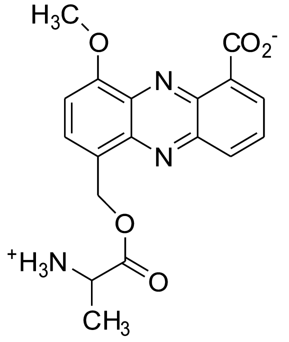
*Figure 7. Chemical structures of Pantocin A (**20**) and B (**21**).*

Eh252 is another strain of *P. agglomerans* that has been shown to produce antibiotics, on a minimal medium, that inhibit the growth of *E. amylovora* (*55*). The production of antibiotics was found to be a crucial factor for the biological control of fire blight by Eh252. Comparison of Eh252 mutant strains that were not inhibitory to *E. amylovora* with wild type Eh252 showed marked differences in their ability to reduce the incidence of fire blight in immature pear fruit assays. The inhibition of *E. amylovora* by Eh252 was prevented in the presence of L-histidine as well as proteinase K indicating that this antibiotic has a peptidic nature and that its potential target lies in the histidine biosynthesis pathway. This antibiotic named MccEh252 is believed to belong to the category of microcins and has been shown to control fire blight in the laboratory and in the field (*54*). Antibiosis has also been shown to contribute to biological control of fire blight by *P. agglomerans* strain Eh252 in orchards (*75*).

Eh1087 strain of *P. agglomerans* produces the broad-specturm phenazine peptide antibiotic D-alanylgriseoleutic acid (AGA) **22** (*63*). AGA has been shown to have *in vitro* activity against several Gram positive and Gram negative bacteria including *E. amylovora* (*76*). An Eh1087 strain (EhΔAGA) was much less effective than the strain producing AGA in preventing stigma colonization by *E. amylovora* (*77*).

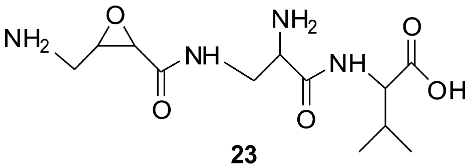
Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019



22

An oxirane containing peptide, 2-amino-3-(oxirane-2,3dicarboxamido)-propanoyl-valine, **23** was isolated from *P. agglomerans* strain 48b/90 (*64*). NMR and MS analysis was used to elucidate the structure of this peptide. The same antibiotic peptide is produced by three different strains of *P. agglomerans*.



23

Magainins and Cecropin-Melitting Hybrids against *E. amylovora*

Several synthetic variants, including hybrids, of the naturally occurring AMPs like magainins, cecropins and melitins have been reported with strong *in vitro* activity against *E. amylovora*. ESF12, a synthetic antimicrobial peptide, mimicking the amphipathic α-helix of magainin, was found to inhibit the growth of *E. amylovora in vitro* with an MIC of 250 μM (*78*). Cecropin B from the silk moth (MW: 3832) and a synthetic analogue of the same named SB-37 (MW:4089) have shown bactericidal activity against *E. amylovora* with MIC values below 15 μM (*79*). BP76 is a cecropin-melittin hybrid linear undecapeptide with an MIC value of 2-5 μM against *E. amylovora* (*80*). BP76 was resistant to proteases and had ED50 of 2.5 μM. BPC194 and BPC198 are the first set of cyclic peptides reported to have strong antibacterial activity against *E. amylovora* (*81*). These peptides, designed and synthesized using a combinatorial chemistry approach, had an MIC of 6-12 μM (BPC194) and 12-25 μM (BPC198) against *E. amylovora* PMV6076 strain and displayed very low cytotoxicity against mammalian cells when used at approximately 100 times the MIC concentrations, implying that they are suitable candidates for field trials at the antibiotic concentrations currently used in such experiments. These peptides have been specifically designed to induce protease resistance by the judicious choice of D-amino acids at specific positions in their sequences and by way of cyclization. Work on the combinatorially synthesized peptides was extended by the incorporation of D-amino acids which resulted in ten peptides with strong inhibitory activity against *E. amylovora*. Representative members from this combinatorial library (BP143 and BP145) containing one D-amino acid at the two and four positions in their sequences were also evaluated in immature pear fruits infected with the bacterium and in *in planta* assays for fire blight control in pear. Results of these assays showed that BP143 had comparable efficacy to streptomycin in controlling fire blight and hence has the potential to be developed as a plant protection product (*82*).

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Conclusions

Antimicrobial resistance is a problem that has existed ever since Sir Alexander Fleming discovered penicillin in the 1940s. Fleming did realize the seriousness of the issue which he addressed in his Nobel lecture where he said '*Penicillin is to all intents and purposes non-poisonous and there is no need to worry about giving an overdose and poisoning the patient. There may be a danger though in an under dosage. ... Moral: If you use penicillin, use enough*'. In the decades that followed, several antibiotics, natural and semi-synthetic, became available that helped to contain these infectious diseases. By the 1980s there was this widespread notion that 'infectious diseases is a thing of the past'. However, infectious diseases came back with a vengeance because of wide spread resistance to conventional antibiotics by the microbes. The message from the WHO director general on the World Health Day 2011 reads '"*In the absence of urgent corrective and protective actions, the world is heading towards a post-antibiotic era, in which many common infections will no longer have a cure and, once again, kill unabated*."

The solution to this public health problem is provided by nature itself in the form of antimicrobial pepitides (AMPs) that act as defense weapons to combat fatal microbial infections (*83*). The AMPs have found potential not only to combat human pathogens, but also as potential solutions in the animal world and to provide protection to infectious diseases of plants.

This chapter has summarized the antimicrobial peptide based compounds reported from various laboratories around the world that have shown potential for chemical (and in some cases biological) control of fire blight. Some of these peptides have also shown broad spectrum antibacterial activity and the ability to inhibit multidrug resistant bacterial biofilms, not only in the fire blight pathogen, but also more problematic human pathogens as well. Even though peptide based molecules have shown promise to tackle the multidrug resistant bacterial biofilms that affect humans, their use in controlling plant pathogens is currently unheard of. Our research in this area has shown the potential of peptide based molecules to control bacterial biofilms amongst plant pathogens, using *E. amyolovora* as a test case. In our research we have used traditional biological screening and analog synthesis methods together with the more modern molecular modeling tools to identify potential targets and develop novel peptide based molecues with antimicrobial activity. The work presented in this chapter on 'inhibiting biofilm formation in *E. amylovora'* is in itself a preliminary account only and is one of the major ongoing research projects in the author's laboratory currently. In future, this class of molecules have the potential to provide the desirable solution to tackle the wide spread public health problem of combating antimicrobial resistance.

Acknowledgments

The author would like to thank Dr. Robin Mitchell for proof reading this chapter. The phytotoxicity and immature pear fruit assays were carried out in the laboratory of Dr. Joel Vanneste, Plant and Food Research Ltd., Hamilton, New Zealand. Part of the research from the author's laboratory reported in this chapter was supported by an Auckland Uniservices grant No. 1078476.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

References

1. Johnson, K. B.; Stockwell, V. O. *Annu. Rev. Phytopathol.* **1998**, *36*, 227.
2. van der Swet, T. *Acta Hortic.* **1996**, *411*, 7.
3. Jenssen, H.; Hamill, P.; Hancock, R. E. *Clin. Microbiol. Rev.* **2006**, *19*, 491.
4. Koczan, J. M.; McGrath, M. J.; Zhao, Y.; Sundin, G. W. *Phytopathology* **2009**, *99*, 1237.
5. Tart, A. H.; Wozniak, D. J. *Curr. Top. Microbiol. Immunol.* **2008**, *322*, 193.
6. Psaltis, A. J.; Wormald, P. J.; Ha, K. R.; Tan, L. W. *Laryngoscope* **2008**, *118*, 895.
7. Singh, P. K.; Parsek, M. R.; Greenberg, E. P.; Welsh, M. J. *Nature* **2002**, *417*, 552.
8. Hancock, R. E. *Expert Opin. Investig. Drugs* **1998**, *7*, 167.
9. Schroth, M. N.; Thomson, S. V.; Hildebra, Dc; Moller, W. J. *Annu. Rev. Phytopathol.* **1974**, *12*, 389.
10. Eastgate, J. A. *Mol. Plant Pathol.* **2000**, *1*, 325.
11. Vanneste, J. L. *Fire blight the disease and its causative agent, Erwinia amylovora*; CABI Publishing: Oxfordshire, 2000; Vol. 1, pp 341−346.
12. Norelli, J. L.; Jones, A. L.; Aldwinckle, H. S. *Plant Dis.* **2003**, *87*, 756.
13. Beaven, P. *New Zealand Herald* **2009**.
14. Wei, Z. M.; Laby, R. J.; Zumoff, C. H.; Bauer, D. W.; He, S. Y.; Collmer, A.; Beer, S. V. *Science* **1992**, *257*, 85.
15. Molina, L.; Rezzonico, F.; Defago, G.; Duffy, B. *J. Bacteriol.* **2005**, *187*, 3206.
16. Psallidas, P. G.; Tsiantos, J. *Fire blight the disease and its causative agent, Erwinia amylovora*; CABI Publishing: Oxfordshire, 2000; p 199.
17. McManus, P. S.; Stockwell, V. O.; Sundin, G. W.; Jones, A. L. *Annu. Rev. Phytopathol.* **2002**, *40*, 443.
18. van der Zwet, T.; Beer, S. V. *Bulletin No. 631*; U.S. Department of Agriculture: Washington, DC, 1992.
19. van der Zwet, T.; Keil, H. L. *Can. J. Microbiol.* **1972**, *18*, 893.
20. Sundin, G. W.; Jones, A. L.; Fullbright, D. W. *Phytopathology* **1989**, *79*, 861.
21. Cooskey, D. A. *Annu. Rev. Phytopathol.* **1990**, *28*, 201.
22. Waksman, S. A. *Mycologia* **1947**, *39*, 565.
23. Goodman, R. N. *Res. Bull. - Mo., Agric. Exp. Stn.* **1954**, *540*, 1.
24. Murneek, A. E. *Phytopathology* **1952**, *42*, 57.

25. Schroth, M. N.; Thomson, S. V.; Hildebrand, D. C. *Annu. Rev. Phytopathol.* **1974**, *12*, 389.
26. Morgan, B. S.; Goodman, R. N. *Plant Dis. Rep.* **1955**, *39*, 487.
27. Paulin, J. P.; Lauchaud, G.; Chartier, R. *Acta Hortic.* **1987**, *217*, 239.
28. Aldwinckle, H. S.; Norelli, J. L. *Acta Hortic.* **1990**, *273*, 391.
29. Bonn, W. G. *Acta Hortic.* **1984**, *151*, 205.
30. Dunegan, J. C.; Kienholz, J. R.; Wilson, R. A.; Morris, W. T. *Plant Dis. Rep.* **1954**, *38*, 666.
31. Hancock, R. E. *Lancet* **1997**, *349*, 418.
32. Hancock, R. E.; Chapple, D. S. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317.
33. Loffet, A. *J. Pept. Sci.* **2002**, *8*, 1.
34. Du Vigneaud, V.; Ressler, C.; Swan, J. M.; Roberts, C. W.; Katsoyannis, P. G. *J. Am. Chem. Soc.* **1954**, *76*, 3115.
35. Bodanszky, M. *Nature* **1955**, *175*, 685.
36. Bodanszky, M. *Int. J. Pept. Protein Res.* **1985**, *25*, 449.
37. Bodanszky, M.; Ondetti, M. A. *Chem. Ind.* **1966**, *1*, 26.
38. Zervas, L. *Z. Naturforsch. B: Chem. Sci.* **1970**, *25*, 322.
39. Zervas, L.; Hamalidis, C. *J. Am. Chem. Soc.* **1965**, *87*, 99.
40. Zervas, L.; Photaki, I.; Cosmatos, A.; Borovas, D. *J. Am. Chem. Soc.* **1965**, *87*, 4922.
41. Merrifield, R. B. *Biochemistry* **1964**, *3*, 1385.
42. Merrifield, R. B. *Adv. Enzymol. Relat. Areas Mol. Biol* **.1969**, *32*, 221.
43. Thayer, A. M. *Chem. Eng. News* **2011**, *89*, 13.
44. Mayo, K. H. *Trends Biotechnol.* **2000**, *18*, 212.
45. Mayo, K. H.; Haseman, J.; Young, H. C.; Mayo, J. W. *Biochem. J.* **2000**, *349* (Pt 3), 717.
46. Mygind, P. H.; Fischer, R. L.; Schnorr, K. M.; Hansen, M. T.; Sonksen, C. P.; Ludvigsen, S.; Raventos, D.; Buskov, S.; Christensen, B.; De Maria, L.; Taboureau, O.; Yaver, D.; Elvig-Jorgensen, S. G.; Sorensen, M. V.; Christensen, B. E.; Kjaerulff, S.; Frimodt-Moller, N.; Lehrer, R. I.; Zasloff, M.; Kristensen, H. H. *Nature* **2005**, *437*, 975.
47. Schroder, J. M.; Harder, J. *Med. Sci. (Paris)* **2006**, *22*, 153.
48. Schroder, J. M.; Harder, J. *Cell Mol. Life Sci.* **2006**, *63*, 469.
49. Jabrane, A.; Sabri, A.; Compere, P.; Jacques, P.; Vandenberghe, I.; Van Beeumen, J.; Thonart, P. *Appl. Environ. Microbiol.* **2002**, *68*, 5704.
50. Ko, K. S.; Norelli, J. L.; Reynoird, J. P.; Boresjza-Wysocka, E.; Brown, S. K.; Aldwinckle, H. S. *Biotechnol. Lett.* **2000**, *22*, 373.
51. Borejsza-Wysocka, E.; Norelli, J. L.; Aldwinckle, H. S.; Malnoy, M. *BMC Biotechnol.* **2010**, *10*, 41.
52. Liu, Q.; Ingersoll, J.; Owens, L.; Salih, S.; Meng, R.; Hammerschlag, F. *Plant Cell Rep.* **2001**, *20*, 306.
53. Liu, Q.; Ingersoll, J.; Owens, L.; Salih, S.; Meng, R.; Hammerschlag, F. *Proceedings of the 4th International Symposium on in Vitro Culture and Horticultural Breeding* **2001**, 95.
54. Vanneste, J. L.; Cornish, D. A. *Acta Hortic.* **2008**, *793*, 391.
55. Vanneste, J. L.; Yu, J.; Beer, S. V. *J. Bacteriol.* **1992**, *174*, 2785.
56. Ishimaru, C. A.; Klos, E. J.; Brubaker, R. R. *Phytopathology* **1988**, *78*, 746.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

57. Mitchell, R. E. *Acta Hortic.* **1993**, *338*, 219.
58. Mitchell, R. E. *Acta Hortic.* **1996**, *441*, 319.
59. Wodzinski, R. S.; Paulin, J. P. *J. Appl. Bacteriol.* **1994**, *76*, 603.
60. Brady, S. F.; Wright, S. A.; Lee, J. C.; Sutton, A. E.; Zumoff, C. H.; Wodzinski, R. S.; Beer, S. V.; Clardy, J. *J. Am. Chem. Soc.* **1999**, *121*, 11912.
61. Winkelmann, G.; Lupp, R.; Jung, G. *J. Antibiot (Tokyo)* **1980**, *33*, 353.
62. Wright, S. A.; Zumoff, C. H.; Schneider, L.; Beer, S. V. *Appl. Environ. Microbiol.* **2001**, *67*, 284.
63. Giddens, S. R.; Feng, Y.; Mahanty, H. K. *Mol. Microbiol.* **2002**, *45*, 769.
64. Sammer, U. F.; Volksch, B.; Mollmann, U.; Schmidtke, M.; Spiteller, P.; Spiteller, M.; Spiteller, D. *Appl. Environ. Microbiol.* **2009**, *75*, 7710.
65. Mitchell, R. E.; Greenwood, D. R.; Sarojini, V. *Phytochemistry* **2008**, *69*, 2704.
66. Augen, J. *Drug Discovery Today* **2002**, *7*, 315.
67. Sarojini, V.; Balaji Rao, R.; Ragothama, S.; Balaram, P. *J. Pept. Sci.* **2010**, *16*, 430.
68. Vijayalekshmi, S.; George, S. K.; Andersson, L. K.; Kihlberg, J.; Baltzer, L. *Org. Biomol. Chem.* **2003**, *1*, 2455.
69. De Zoysa, G. R. J.; Mitchell, R. E.; Sarojini, V. *Poster presentation at the 12th International Workshop on Fire Blight, Aug 2010, Warsaw, Poland* **2010**.
70. Scott, H. V.; Gero, A. M.; Osullivan, W. J. *Mol. Biochem. Parasitol.* **1986**, *18*, 3.
71. Mitchell, R. E.; Teh, K. L. *Org. Biomol. Chem.* **2005**, *3*, 3540.
72. Mitchell, R. E. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1910.
73. De Zoysa, G.; Mistry, M.; Washington, V.; Lewis, G.; Mitchell, R. E.; Sarojini, V. *Acta Hortic.* **2011**, *896*, 541.
74. Vanneste, J. L.; Yu, J.; Harper, G. E.; Perry, J. H. *Acta Hortic.* **1996**, *411*, 303.
75. Stockwell, V. O.; Johnson, K. B.; Sugar, D.; Loper, J. E. *Phytopathology* **2002**, *92*, 1202.
76. Giddens, S. R.; Bean, D. C. *Int. J. Antimicrob. Agents* **2007**, *29*, 93.
77. Giddens, S. R.; Houliston, G. J.; Mahanty, H. K. *Environ. Microbiol.* **2003**, *5*, 1016.
78. Powell, W. A.; Catranis, C. M.; Maynard, C. A. *Mol. Plant-Microbe Interact.* **1995**, *8*, 792.
79. Mourgues, F.; Brisset, M. N.; Chevreau, E. *Plant Sci.* **1998**, *139*, 83.
80. Ferre, R.; Badosa, E.; Feliu, L.; Planas, M.; Montesinos, E.; Bardaji, E. *Appl. Environ. Microbiol.* **2006**, *72*, 3302.
81. Monroc, S.; Badosa, E.; Besalu, E.; Planas, M.; Bardaji, E.; Montesinos, E.; Feliu, L. *Peptides* **2006**, *27*, 2575.
82. Guell, I.; Cabrefiga, J.; Badosa, E.; Ferre, R.; Talleda, M.; Bardaji, E.; Planas, M.; Feliu, L.; Montesinos, E. *Appl. Environ. Microbiol.* **2011**, *77*, 2667.
83. Zasloff, M. *Nature* **2002**, *415*, 389.

Downloaded by OHIO STATE UNIV LIBRARIES on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch019

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Chapter 20

Thionins - Nature's Weapons of Mass Protection

Svetlana Oard,*,[1] Jong Hyun Ham,[2] and Marc Alan Cohn[2]

[1]School of Plant, Environmental, and Soil Sciences, Louisiana State University Agricultural Center, Baton Rouge, LA 70803
[2]Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803
***E-mail: SOard@agcenter.lsu.edu**

Fungal and bacterial diseases cause millions of dollars of crop damage, presenting an ongoing challenge for farmers, as well as undermining food safety. A broad-range protection system against microbial phytopathogens is needed to reduce or even eliminate crop yield dependence upon pesticides, which increase farmers' fixed costs globally or are not available/affordable to smallholder farmers in developing countries. While breeding crop resistance to multiple microbial diseases has been a desirable goal, numerous attempts to develop resistance by conventional breeding methods have had limited success. Thionins, a class of plant antimicrobial peptides, are excellent candidates for developing a broad-range plant defense system. They exhibit broad activity against bacteria and fungi, are effective at low concentrations, and rapidly damage microbial cells. Thionins act on membranes, greatly reducing the development of the pathogen resistance. Seed-specific thionins from wheat and barley are of particular interest because they meet requirements for genetic engineering of antimicrobial resistance in important crops. While reliable protection against microbial pathogens has been obtained in several plant species transgenically expressing leaf-specific thionins, inconsistent results have been reported for seed-specific thionins, even though high antimicrobial activity of thionins occurs in vitro. Despite extensive study, the natural mechanisms by which plants mobilize thionins to inhibit bacterial and fungal pathogens are ill-defined. Here, we

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

summarize the considerable evidence that thionins are suitable for developing a reliable broad range antimicrobial defense system for agronomically important crops. New breakthroughs in understanding of thionin function in plant cells, and in particular the critical role of thionin signal peptide structure for regulation of thionin activity during its processing and transport in leaf tissues, should enable development of a thionin-based crop protection system.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Introduction

The world population is currently 6.8 billion (*1*), and in spite of the ‘green revolution’, which doubled the world’s major crop production between mid-1950s and mid-1990s, more than 2 billion people in developing countries are still suffering from hunger or malnutrition (*2*). The worldwide ‘agflation’ experienced in 2007-2008 clearly demonstrated how the food supply crisis can hurt the global economy and cause severe political and social problems in many developing nations. Arable lands are rapidly decreasing worldwide due to growing human population, urbanization, and global climate changes. Moreover, about 25-50% crop yield losses occur every year from diverse pests, including arthropods and microbes causing plant diseases. Thus, successful pest/disease control is essential for maintaining stable food production (*2*).

All foods are ultimately the products of photosynthesis. So, regardless of crop species, leaves should be the primary part to be protected from pathogens and insects. Most major yield-limiting plant diseases are foliar diseases. Powdery mildew, downy mildew and rust are well-known foliar diseases caused by fungal pathogens, and they are severe airborne disease problems in cereals, vegetables and fruits. Therefore, reinforcement of leaf tissues using biotechnology would efficiently enhance the resistance of crops to these foliar diseases.

Each crop species can be damaged by many different diseases and pests throughout its life cycle. In common practice, diverse chemicals effective against different pathogens and insects are applied multiple times to fully protect a crop from all possible diseases and pests, which requires enormous economic and environmental costs. Moreover, chemical control measures are not available for numerous diseases (i.e. viral and bacterial diseases), for which disease control is dependent solely on disease-resistant varieties. Disease-resistant crops are much more valuable and useful in many developing countries because pesticide availability and affordability are limited.

As each species can be attacked by a wide range of pathogens, it is important to develop crops with broad resistance to diverse pathogens. Development of broad disease resistance will save time and resources that would be required for developing resistance for specific pathogens individually. While breeding crops resistant to multiple microbial pathogens has been a great desire, the variation in bacterial and fungal populations has made it difficult to identify stable determinants of crop resistance (*3*, *4*). Numerous attempts to develop resistance by conventional breeding methods have had limited success. A

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

promising approach towards this goal is identification and introduction of novel genes encoding effective resistance against bacterial and fungal pathogens into important crops. In particular, introduction of antimicrobial peptides through plant transformation could create crops resistant to a wide range of bacterial and fungal pathogens (*4–7*).

Because thionins, highly basic plant antimicrobial peptides, have a broad-spectrum anti-microbial activity (*8–10*), enhancement of thionin production and accumulation in plants will be an excellent strategy to promote broad disease resistance of crops against diverse bacterial and fungal pathogens. Broadly resistant varieties can not only prevent yield losses due to bacterial and fungal diseases, but also expand the boundaries of possible growing areas of economically important crops, which are limited by disease problems. Introduction of crops with improved disease resistance will significantly reduce the costs for chemicals, which will also save our environment. In this review, we summarize the role of thionins in plant protection and our progress to create a broad-range, thionin-based protection system against microbial phytopathogens.

Engineering Disease Resistance in Crops

Plant diseases caused by fungi and bacteria limit stable food production worldwide (for examples see Tables –II and I-III in (*11*)). A crop is attacked by a diverse array of pathogenic microorganisms having different virulence mechanisms (*12*, *13*). Each plant possesses different defense systems for surviving attacks from different types of pathogens. When a plant interacts with various pathogens, it activates appropriate systems modulating specific signaling pathways for defense (*12*, *14*). Defense systems have been intensively studied, and many signaling pathways for defense and their components have been revealed (*15–17*). In general, salicylic acid (SA)-mediated signaling pathways are involved in plant defense against biotrophic fungal and bacterial pathogens, while jasmonic acid (JA)/ethylene-mediated pathways are for the defense against necrotrophic pathogens (*12*, *18*).

However, these plant defense systems are continuously overcome by rapid adaptation of pathogens to previously resistant hosts. Many pathogens are known to produce virulence factors that suppress various plant defense signaling pathways (*17*, *19*, *20*). Plants are forced into “arms race” with microbial pathogens. In addition, some major defense signaling pathways are mutually antagonistic, preventing the activation of all defense systems at one time (*12*). Therefore, development of crops having durable and broad-spectrum resistance to all types of pathogens is very challenging. In this sense, it is an ideal approach to introduce an element having lethal activity toward multiple pathogens through genetic engineering technology.

Strategies for Engineering Resistance in Crops

Currently, strategies for management of microbial diseases in crops include crop management, chemical control, classical breeding, and genetic engineering.

To date, broad spectrum pathogen resistance has not been generally possible with any combination of these approaches.

While genetic engineering allows fast and targeted introduction of specific genes, eliminating the major drawbacks of the classical breeding, this strategy has been associated with food safety, environmental, and economic concerns as well (for reviews see (*8*, *21*, *22*)). For example, an introduced gene might be toxic to human and animal consumption directly or through modifying metabolic pathways in plant cells. A foreign protein may cause allergies in humans or render toxicity to beneficial microbes or insects. The spread of a transgene to wild relatives of a genetically modified crop via cross-pollination could increase the competitiveness of weeds. Widespread disease resistant crops could trigger emergence of resistant microorganisms, which could overcome natural immune barriers of plants and even humans. In addition, overexpression of a transgene can deplete particular metabolic pathways, consequently decreasing yield. These concerns hold true for classical breeding as well. Importantly, genetic engineering allows a tighter control of these undesirable consequences through introduction of single genes, unlike the classical breeding. Knowledge of a gene function and careful selection of candidate genes for transformation may eliminate these concerns.

There are other concerns that are specific to genetic engineering. The use of antibiotic resistance genes as selection markers of transgenic plants raised issues about unpredictable biohazards to human health and ecosystems from introducing antibiotic resistance into food supply and environment. These concerns were fueled by a possibility of horizontal gene transfer from transgenic plants to prokaryotes. Horizontal gene transfer, which can be enabled by several mechanisms, is common between prokaryotes (for review see (*23*)). Fortunately, recent studies showed that horizontal gene transfer from transgenic plants to prokaryotes is at least 10^{14} times lower than between bacteria and therefore is not expected to influence prokaryotic evolution. Furthermore, new transformation tools have been developed that allow excision of unwanted selectable markers after transformation or full replacement of antibiotic resistance genes (*24*).

Can we find genes for genetic engineering that can alleviate or even eliminate these concerns and provide the desirable benefits? To answer this question, we have to define the ideal characteristics of a gene for engineering resistance to microbial diseases. First, a trangene product must be non-toxic for human consumption and animal feed as well as non-allergenic. The most evident is to use genes that are already abundantly expressed in edible parts of common food crops when possible. Second, transgenic plants must be safe for beneficial insects and microbes. A selective targeting may be achieved by using tissue specific promoters. For example, expression of a gene with a broad range of activity under a leaf-specific promoter can protect microorganisms in rhizosphere. Promoters can be designed to prevent accumulation of transgenic proteins in pollen to protect beneficial insects. Another way is to use genes encoding defense proteins that are bound within plant tissues and thus prevented from release into rhizosphere with root exudates.

Third, development of resistant crops using an ideal gene has to be economically feasible. A trait has to be encoded by a single dominant gene characterized by Mendelian inheritance and stable transformation that is not prone

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

to gene silencing. As each species can be attacked by a wide range of pathogens, it is important to develop crops with broad resistance to diverse diseases. Another important characteristic is minimal negative impact on crop yield. Because overexpression of a foreign gene can drain cellular resources from building yield, genes that are effective at minimal or moderate levels of expression to achieve desirable resistance are highly preferable. Rendered resistance must be durable to withstand high selection pressure of phytopathogens to overcome resistance. To meet the challenge of quickly evolving microbial phytopathogens, we need to develop defense mechanisms that are less likely to be overcome by microorganisms.

Pathogens target multiple steps in the plant innate immune system. In fact, microbial pathogens evolved offense mechanisms targeting the plant immune system at every level (*17*, *20*, *25*). Currently, biotechnological strategies for enhancing antimicrobial resistance in crops include direct inhibition of pathogen physiology, the regulation of the natural induced host defense (enhancing pathogen recognition or defense signaling), interfering with virulence strategies of microbial pathogens, and pathogen mimicry for pathogen-derived response (for reviews see (*3*, *21*)). Each strategy has its own pros and cons. We show below that the genes encoding thionins, plant antimicrobial peptides, are excellent candidates for developing crop resistance to a wide range of bacterial and fungal pathogens.

Development of a thionin-based crop protection strategy can deliver far reaching benefits of increasing yields by minimizing losses to microbial diseases, reducing costs for pesticides, and reducing environmental pesticide contamination. A thionin-based crop protection system, especially antifungal resistance, requires effective levels of antimicrobial peptide production and accumulation of the biologically active peptide in the path of s pathogen. Recent breakthroughs in understanding of thionin function in plant cells open opportunities to use this gene to develop commercial crops with enhanced antibacterial and antifungal resistance.

Thionins – Part of a Plant Defense System

Antimicrobial peptides are important components of non-specific host defense systems and innate immunity in insects, amphibians, plants, and mammals (*5*, *7*, *26–28*). However, many antimicrobial peptides exhibit antibacterial activity, but have little to no antifungal activity (*5*, *29*, *30*). Five- to twenty–fold larger concentrations of antimicrobial peptides are required to inhibit fungal cells as compared to bacterial cells (*31–33*).

In contrast, thionins, a family of highly basic plant antimicrobial peptides, have broad spectrum antibacterial as well as antifungal activities (*9*, *34–36*). Thionins are low molecular weight peptides present in leaves, seeds, and flowers (*37–40*). Thionin genes can be expressed constitutively in seeds and seedlings or are inducible by methyl jasmonate and pathogenic fungi in leaves.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Antimicrobial Activity

Microbial growth inhibition by thionins is concentration dependent, which is similar to linear amphipathic antimicrobial peptides. However, thionin antifungal activity surpasses activity of well-known linear antimicrobial peptides. Some of most studied thionins are β-purothionin (βPTH) and α-hordothionin (αHTH) from the wheat and barley endosperm, respectively. The antibacterial and antifungal activities of these peptides exceed several well known linear antimicrobial peptides in antifungal activity *in vitro* (*36*, *41*, *42*). When twelve natural and synthetic antimicrobial peptides were directly compared in a fungal growth inhibition bioassay to antifungal antibiotics (nystatin and nikkomycin Z), βPTH showed the strongest inhibitory activity with levels similar to those of the antibiotics (Table I) (*42*). βPTH was also the most stable against proteolytic degradation when added to liquid cultures of *Rhizoctonia*.

Table I. Inhibitory concentrations of antimicrobial peptides and antibiotics tested against *Rhizoctonia solani* (fungal pathogen of rice) (*42*)

Compound	*Source*	*Residues*	*IC_{50} * (μmol)*	*MIC † (μmol)*
Cecropin B	*Cecropia* moth	35	4.6 ± 0.3‡	9.8
Magainin II	African frog	23	15.7 ± 0.9	30.2
Melittin	Honey bee	26	7.1 ± 0.6	13.1
βPTH	Wheat	45	1.1 ± 0.4	5.0
D4E1	Synthetic	17	4.5 ± 0.3	8.7
D2A21	Synthetic	23	6.5 ± 1.9	18.4
pep11	Synthetic	10	11.2 ± 2.1	24.1
phor14	Synthetic	14	19.2 ± 2.9	37.9
phor21	Synthetic	21	8.4 ± 2.0	18.6
Nikkomycin Z	*Streptomyces*	antibiotic	0.9 ± 0.1	4.5
Nystatin	*Streptomyces*	antibiotic	1.1 ± 0.3	5.2

* IC_{50}, concentration that inhibits 50% and † MIC, minimum concentration that completely inhibits growth of fungal cells; ‡ 95% confidence interval.

Most proposed mechanisms of microbial growth inhibition by linear peptides and thionins invoke interaction with phospholipids to cause membrane instability (*10*, *43–46*). Although different properties, such as inhibition of protein synthesis, vary among these groups of peptides, they share one common property: the ability to permeabilize membranes (Fig. 1). Moreover, fungal or bacterial growth inhibition correlates with membrane permeabilizing activity for both linear amphipathic antimicrobial peptides and thionins (*30*, *41*, *42*). Noticeable changes in membrane integrity were observed at concentrations of $\geq$0.5 emol l^{-1} for βPTH, 2 μmol l^{-1} for cecropin B and melittin, and 8 μmol l^{-1} for magainin II. These data correlated with antimicrobial activity of these peptides (Table I). Fluorescence studies of βPTH uptake by fungal hyphae showed a rapid increase in membrane permeability within 1 min, reaching plateau values in nearly 10 min (*42*). This evidence indicates that membrane permeabilization is a primary cause of inhibiting growth of microorganisms.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

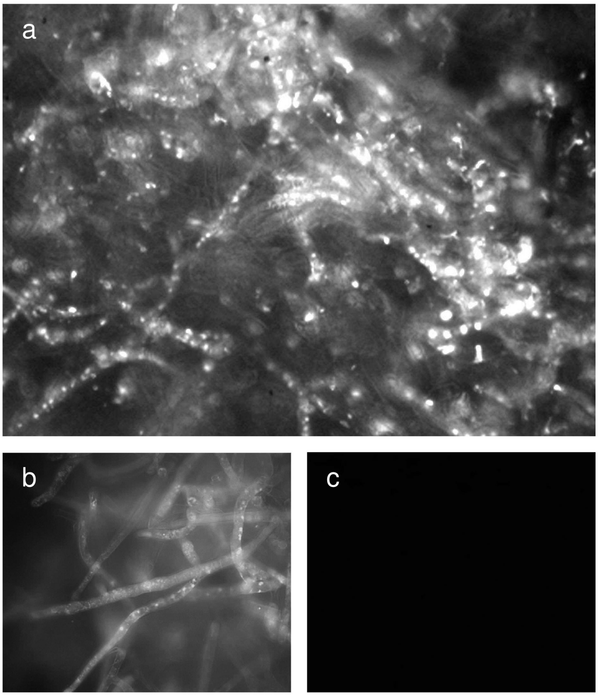


Figure 1. Membrane permeabilization of a fungal phytopathogen Rhizoctonia solani by antimicrobial peptides βPTH and cecropin B. Cells were supplemented with 0.5 μM nuclear stain Sytox Green and (a) 4 μM βPTH, (b) 16 μM cecropin B, or (c) H2O and incubated for 10 min before fluorescent microscopy.

The mode of peptide uptake involves both endocytic and non-endocytic paths, which are temperature-sensitive and –insensitive processes, respectively (*47*). βPTH was added to pre-chilled cells in that study, and the detection of membrane permeabilization 1 min after peptide addition indicated a temperature-insensitive non-endocytic uptake. Rapid uptake of Ca^{2+} was also reported for αHTH when it was added at an inhibitory concentration to a fungal cell suspension (*48*). Because thionins act by permeabilizing microbial membranes, there is less likelihood that target microbes will develop resistance to these peptides (*7*, *46*).

Not only do thionins exhibit high antibacterial and antifungal activity *in vitro*, but they also render antimicrobial resistance *in planta*. Cell-wall-bound thionins accumulated in high amounts at penetration sites of a resistant, but not a susceptible barley cultivar when infected with a fungal pathogen causing powdery mildew (*49*). Thionin overexpression also increased resistance to fungal and bacterial pathogens. For example, overexpression of an endogenous thionin (encoded by the *Thi2.1* gene in *Arabidopsis thaliana*) enhanced plant resistance to *Fusarium oxysporum* (*37*). Exogenous expression of the same gene in tomato also enhanced resistance to bacterial wilt and Fusarium wilt (*50*). Overproduction of an oat cell-wall-bound thionin in rice enhanced resistance to seed-transmitted bacterial diseases (*51*). Conversely, silencing of a thionin gene (*PR13/Thionin*) reduced antimicrobial resistance of a naturally resistant *Nicotiana attenuata* to *Pseudomonas syringae pv. tomato DC3000* (*52*). These results encourage further work to develop strategies to effectively produce thionins in disease susceptible crops.

Structure

Thionins are 45-47 amino acids long, highly basic, and resistant to various environmental conditions (*10*, *38*). Secondary structure of thionins is conserved and consists of a β-sheet and a double α-helix core bound by three or four disulfide bridges. The thionin family consists of five types of peptides (*10*) and can be divided into two subfamilies according to the number of disulfide bonds. βPTH belongs to a thionin subfamily containing type I and II basic thionins with four disulfide bonds (4DSB subfamily). Representative members of this subfamily are α-and β-purothionins from wheat seeds, α- and β-hordothionins from barley seeds, barley leaf thionins (DB4, BTH6, and DG3), and the oat leaf thionin Asthi1 (*34*, *53*, *54*). The subfamily with three disulfide bonds (3DSB subfamily) includes representative thionins such as Arabidopsis thionins Thi2.1 and Thi2.2, crambins, and seed-specific thionins from *Abyssinian cabbage*.

The two subfamilies share only 15 to 35% sequence homology where six cysteine residues and K1, R10, and Y13 are highly conserved. Substitutions of K1 and Y13 have been associated with loss of activity (*10*). The 4DSB subfamily shares a considerably high sequence homology determined by three conserved motifs. The N-terminal motif KSCC and the C-terminal YPK carry K1, S2, and K45, which belong to a thionin phospholipid-binding site that was identified by crystallography (Fig. 2) (*55–57*). The third motif RNCYNxxCR, amino acid residues 9 through 17 in βPTH, carries R10 and Y13, which form the center of the phospholipid-binding site. Computational analysis showed that R17 and Q22, which are found in hordo- and purothionins, also participate in the binding site as well as residues of the L1 loop (*58*). Participation of additional residues in the binding site may explain high antimicrobial activity of these thionins.

The phospholipid-binding site is located in the groove that is formed by the arm and stem at the inner corner of the global Γ fold of thionins (Fig. 2). The main contributors of the phospholipid-binding site include K1, S2, R10, Y13, and R17 (*57*). It was recently revealed that the opposite side of the thionin global fold contains a functional R30 hydrogen-bonding network that plays an important role

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

in membrane permeabilizing activity (*59*). The central part of the R30 regulating network consists of two charged residues tightly bound to each other by two disulfide bonds. This structure creates a spring that regulates opening of a water channel (*59*, *60*). The R30 networks of βPTH and αHTH are D42-K5-R30-N27 and G42-R5-R30-G27, respectively. The majority of thionins with four disulphide bonds have amino acid residues in the corresponding positions, which should form similar regulating networks. For example, in the oat avenothionin α (D42-K5-R30-T27) (*61*), *Tulipa gesneriana* thionin Class1 (D43-R5-R31-A28) (*62*), and *Pyrularia pubera* thionin (PpTH) (D44-R5-K29-D32) (*63*). Most recently, computational analysis showed that hordo- and purothionins acquired an auxiliary network R(K)5-K32-K23-Q22-Y13 that connects the R30 regulating network with the phospholipid-binding site. These networks, as well as the additional phospholipid-binding capacity, suggest functional advantages and sophistication of hordo- and purothionins.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

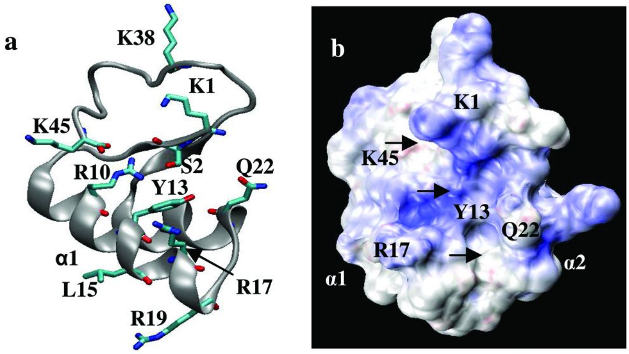


Figure 2. Tertiary structure of a representative thionin αHTH. (a) The secondary structure is shown with selected residues as licorice. The phospholipid-binding site includes K1, R10, Y13, and R17. (b) The peptide surface (same orientation). A large positive electrostatic potential (blue shading) is concentrated on the phospholipid-binding site. α1 and α2, the α1 and α2 helices, respectively. Arrows indicate the groove. (see color insert)

Thionin Toxicity and Allergenicity

The ideal plant protection system should not affect the environment except for pathogenic target organisms. The seed-specific thionins, such as βPTH and αHTH, are safe for humans and animals when ingested, as they are found in abundance in wheat and barley flour. Because thionins, which are only 45-47 residues long, contain nearly 20 trypsin and pepsin cleavage sites (http://ca.expasy.org/tools/peptidecutter/), they are quickly hydrolyzed in a stomach. Furthermore, these peptides are irreversibly inactivated in mammalian blood by mono- and divalent metal ions K^+ Na^+, Ca^{+2}, and Mg^{+2} (*35*, *41*, *64*). In vitro studies showed that two to five mM Ca^{2+} completely blocks activity of

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

βPTH *in vitro*, while 50 mM concentrations of monovalent ions like Na^+ and K^+ are necessary for inactivation (*41*, *64*). Thus, cation concentrations two times lower than in mammalian blood irreversibly inactivate thionins. Experiments demonstrating toxicity of thionins to mammalian cells were conducted under artificial conditions with low ionic strength or even chelating agents to remove cations (*64*, *65*). Thionins could be toxic locally only if injected directly into tissues in high concentrations but not when ingested (*66*). Insects are also not affected upon ingestion of thionins (*52*, *67*). Localization of thionin expression to leaf tissues should provide a spatial separation from beneficial soil mycorrhizal microorganisms. Thionins bind to components of the plant cell wall (*68*, *69*) that prevent them from leaching into soil. Thus, plants transgenically expressing βPTH and αHTH in leaves should only impact pathogenic microorganisms attacking leaf tissues - a highly desirable feature.

A Mechanism of Antimicrobial Activity

There is a growing consensus that thionins inhibit microorganisms by permeabilizing membranes (*10*, *44*, *57*, *60*). As shown by numerous *in vitro* and *in vivo* experiments, thionins permeabilize the bacterial, fungal, mammalian, and plant membranes under low ionic conditions (*8*, *9*, *70*). Thionins bind to the membrane components *in vitro*, especially, to the negatively charged phospholipid phosphatidylserine. The latter is present in bacterial, fungal, mammalian, and plant membranes. PpTH binds to phosphatidylserine and phosphatidic acid *in vitro* (*57*). This thionin effectively solubilizes phosphatidylserine and phosphatidic acid into the aqueous phase from the organic phase, which suggests the formation of a proteolipid complex. In fact, binding is so strong that the crystal structures of both βPTH and α1-purothionin contain the phosphate and the glycerol moiety in the phospholipid-binding sites (*55*, *56*). None of these moieties was present in the crystallization solutions. Evidently, these molecules were remainders of phospholipids that thionins bound to during purification from wheat seeds. In addition, studies of model unilamellar vesicles differing in lipid composition indicated that thionins permeabilize membranes only when the vesicles contain negatively charged phospholipids (*71*). Similarly, no solubilization into the aqueous phase was observed for phosphatidylglycerol and phosphatidylcholine, which are neutral (*57*).

Membrane permeabilization is accompanied by a variety of other effects, such as influx of Ca^{2+} ions and activation of phospholipase A_2 (for review, see (*10*)). These effects are caused by switching defense mechanisms in response to disturbed membrane integrity and cell lysis.

Thionins share the ability to permeabilize membranes with linear amphipathic cationic antimicrobial peptides for which several models of membrane permeabilization have been proposed (for review see (*72*). One of most popular is the carpet model proposing that peptides self-associate in a ‘carpet-like’ manner on the membrane surface. Such in-plane alignment was demonstrated for the antimicrobial peptides, magainines and cecropins. Thionins insert into the membrane bilayer modifying the lipid packing (*44*, *73*). The electrostatic

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

interactions are followed by hydrophobic interactions. However, NMR measurements showed that thionins dissociate into monomers upon interaction with membrane bilayer (*57*).

Three mechanisms of membrane permeabilization by thionins were proposed so far: formation of ion channels (*44*), phospholipid withdrawal (*57*), and a water-selective pore (*60*). Thionins display properties that could be partially explained by formation of ion channels. βPTH, α_1-purothionin, and α_2-purothionin form cation-selective ion channels in artificial lipid bilayers and in the plasmalemma of rat neurons and murine cells (*64*, *74*). Membrane composition affects properties of ion channels. The presence of phosphatidylserine increases signals, which are typical for small protein channels. Other studies with βPTH in dimyristoylphosphatidylglycerol (DMPG) membranes also suggested that interactions of βPTH with the membrane bilayer are consistent with formation of protein channels rather than membrane disruption (*44*, *73*). However, fluorescent probes, which are too large for penetrating through ion channel pores, were released from lipid vesicles in the presence of α-purothionin (*71*). Lysis of yeast cells was observed after addition of 4 μM αHTH in our laboratory (unpublished data). Only cell debris could be found under a fluorescent microscope after 30 min of incubation. These results suggest destabilization and disruption of the membrane, consistent with the fact that α-helices of thionins are too short to span the entire lipid bilayer, and therefore, would not form true ion channels.

The phospholipid withdrawal model for membrane permeabilization by thionins has been proposed based on experimental data showing the ability of PpTH to transfer negatively charged phospholipids from the organic phase to the aqueous phase (*57*). According to this model, thionins bind to negatively charged phospholipids and then withdraw phospholipids, causing solubilization and lysis of the membrane. This model explains membrane disruption but not formation of ion channels at low concentrations of thionin. Molecular docking of phosphatidylserine and its derivatives into the phospholipid-binding site of αHTH showed that not only the phosphate group and glycerol moiety bind to the phospholipid-binding site, but in addition the serine moiety and one acyl chain fit into the thionin groove (*60*). The phospholipid headgroup bound in the groove can become "locked" between the side chains of basic residues. Such a strong binding, where the upper half of the phospholipid is almost engulfed by the peptide, could explain solubilization of phosphatidylserine by PpTH observed experimentally.

The minimal structural requirements for thionin permeabilization activity were determined by testing several truncated PpTH peptide derivatives (*75*). A minimal motif in PpTH with a retained antimicrobial activity consisted of only the antiparallel double α-helix core. Preservation of two disulfide bonds linking the α-helices was crucial for maintaining antimicrobial activity. The minimal motif included the conserved residues R10, Y13, and R17, which belong to the phospholipid-binding site, as well as a residue that corresponds to R30 in βPTH and αHTH (*55*, *56*). An important role of Y13 for antimicrobial activity was directly demonstrated upon iodination of the Y13 hydroxyl group (*65*, *76*, *77*). The βPTH Y13 was inaccessible to deuteration after the peptide was added to the membrane bilayer, suggesting that the portion of βPTH containing the tyrosine

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

residue inserts into the membrane hydrophobic core (*44*). Increased antimicrobial activity was detected in the PpTH D32R variant, where R32 corresponds to R30 in βPTH and αHTH (*75*).

An advanced computational analysis recently demonstrated that the sophisticated tertiary structure of αHTH forms functional dynamic networks tuned by evolution to permeabilize and disrupt microbial membranes (*58–60*, *78*). Nature stepped beyond a single mechanism of membrane permeabilization, as it is known for aliphatic antimicrobial peptides, combining together elements of several models into one multifaceted process to create a perfect molecular tool for membrane disruption.

Molecular dynamics (MD) simulations revealed that anions trigger opening of a water-selective pore inside the αHTH α-helix core (*58*, *60*). This channel contains water-selective filters and gates that are similar to those found in aquaporins, proteins forming water channels in membranes (*79*). Thionins and aquaporins utilize the same principles of selection through charge and size exclusion. The αHTH pore profile consists of three cavities (Fig. 3). The pore mouth leads into the α2 C-end cavity, which is positively charged, barely large enough to fit one water molecule, and is similar to the aquaporin aromatic/arginine selectivity filter (*79*). For αHTH, the average interaction energy between the internalized water and the α2 C-end cavity is very similar to the interaction energy between water and protein at the aquaporin aromatic/arginine selectivity filter. The high electrostatic interaction energy inside the channel at the entrance into the αHTH pore suggests that the α2 C-end cavity serves as a "water sink" attracting water inside the pore. A relatively spacious central cavity is lined mostly by the highly conserved residues, Y13 and six cysteines. The central cavity is connected to the dynamic α1 C-end cavity via the Y13/R17 constriction site, which resembles the aquaporin NPA motif. Two gates, at the Y13/C25 and Y13/R17 constriction sites, involve conserved residues of the phospholipid-binding site. Binding of a phospholipid should pull away the side chains of Y13 and R17 to open the gates.

The thionin size, shape, and the distribution of electrostatic potential on the peptide surface almost precisely control positioning of thionin inside the membrane bilayer. Thionin inserts as a wedge, cutting into one leaflet of the membrane bilayer with the α1/α2 hydrophobic region pointing down and nearly reaching the bilayer center (Fig. 4). The phospholipid-binding site strongly binds a negatively charged phospholipid headgroup and a portion of one acyl chain to anchor the peptide inside membrane. Interaction with the bound phospholipid and negatively charged groups of surrounding phospholipids triggers opening of the water-selective pore. The pore mouth is positioned at the headgroup/hydrocarbon boundary of the leaflet, and the high electrostatic potential attracts water inside the α2 C-end cavity. Water molecules penetrate into the α1 C-end cavity, which is hidden underneath the peptide in a void at the bilayer center. When water molecules become crowded inside the α1 C-end cavity, they contact surrounding acyl chains of phospholipids, triggering a chain reaction of repulsive interactions. Strong repulsive interactions fueled by dynamic properties of thionin lead to expulsion of water from the bilayer center and temporary water defects (*80*).

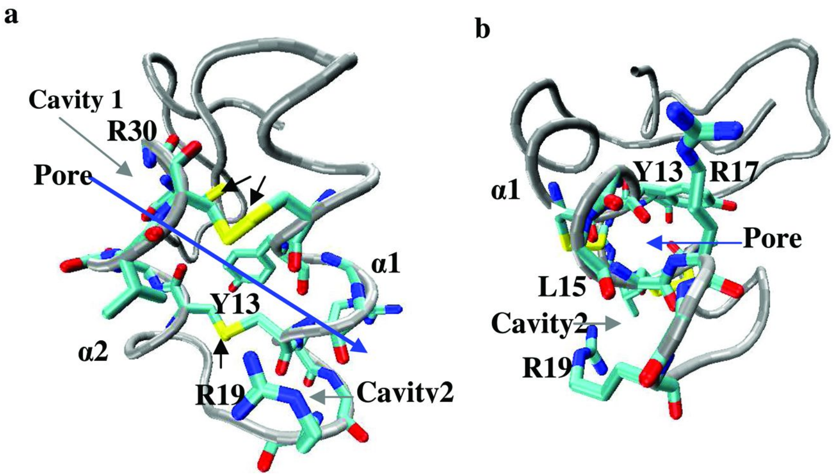


Figure 3. Thionin presents a water channel (adapted from (60). (a) A water permeable pore runs through α-helical core of αHTH starting in cavity 1 and ending in cavity 2. Selected residues forming pore walls are shown as licorice and three disulfide bonds indicated by black arrows. (b) View of the pore from cavity 2. All residues forming pore walls are shown as licorice and the end of the pore is aligned with the pore mouth at cavity 1 to demonstrate the unblocked pore opening in the peptide core. α1 and α2, α-helices α1 and α2, respectively. (see color insert)

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Next, water starts to penetrate into the pore again and the process repeats. Periodicity of this process produces a channel-like activity observable at low thionin concentrations. This mechanism also explains penetration of large molecules that can pass through the membrane bilayer via temporary water defects. With increased thionin concentrations, local water defects can merge, causing membrane disruption.

This mechanism of membrane permeabilization by thionins incorporates the phospholipid-binding site, the R30 regulating network, electrostatic/hydrophobic interaction, and monomeric state of thionin in membranes, explaining channel-like activity as well as membrane disruption. The mechanism holds for the 4DSB subfamily with a few exceptions. However, membrane permeabilization activity has not been confirmed for the majority of thionins deposited in the National Center for Biotechnology Information (NCBI) databases, and some thionins maybe inactive. For example, crambin, which has substitutions K1T and Y13F, shows no activity (*81*). The members of the 3DSB subfamily - while preserving the key residues of the phospholipid-binding site - K1, R10, Y13 - lack Q22, K45, or even S2 and R17, and the R30 regulating network. This fact implies that puro- and hordothionins acquired important additional mutations that brought the structure/function relationships onto a higher level of sophistication.

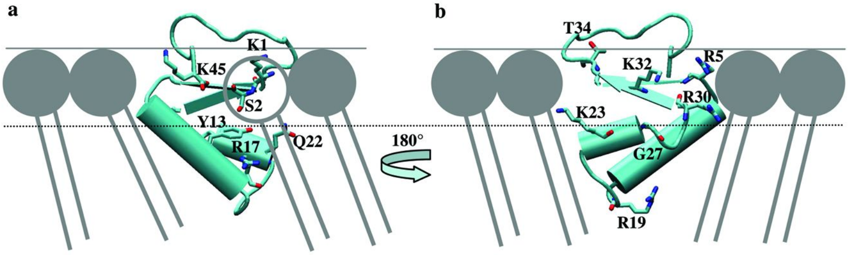


Figure 4. A scheme of interactions between αHTH and a bilayer leaflet (60). (a) A phospholipid molecule is bound to the phospholipid-binding site in the groove of the global fold. The headgroup of the bound phospholipid is shown transparent to illustrate the phospholipid-binding site. (b) On the opposite side, the pore mouth at the α2 C-end is positioned at the interface between hydrophilic headgroups and hydrophobic acyl chains while the stem consisting of α1 and α2 helices is inserted into the hydrophobic core of the membrane bilayer. Selected residues are shown as licorice. The solid line represents a surface of the membrane bilayer. The dotted line runs through the middle of the pore mouth and the glycerol-binding site. (see color insert)

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Endogenous Activity of Thionins *in Planta*

Regulation of Thionin Expression

Thionins are expressed in leaves, seeds, and flowers. The *A. thaliana Thi2.2* transcripts have low basal levels in seedlings and rosette leaves and display circadian variation (*82*). The *A. thaliana Thi2.1* transcripts are present in rosette leaves and at high levels in flowers. In addition, the *Thi2.1* gene is inducible by JA, silver nitrate, and necrotrophic fungi in seedlings but not by SA, the typical elicitor of pathogenesis related proteins (PR) (*37*, *39*). Compared to *PR-1* and *PR-5*, the induction of *Th2.1* is more pronounced. An Arabidopsis mutant *cexI* with a JA-responsive phenotype shows high levels of *Thi2.1* expression, while *Thi2.1* is not inducible in a *coiI* mutant with JA-insensitive phenotype (*83*).

Thionin genes are induced after pathogen attack in barley (*34*, *49*, *84*). In barley and rice, thionin is expressed at high levels only during first 24 hours after germination in the light (*40*, *85*). JA, as well as darkness, extends expression of thionin. A signaling pathway maintaining high thionin levels in the dark is JA independent (*40*). These data indicate complex transcriptional regulation of thionin genes that differs from signaling pathways regulating transcription of the majority of pathogenesis-related genes, which are SA dependent.

A structural gene for thionins includes regions encoding a signal peptide, a mature thionin domain, and a C-terminal acidic protein. Thionins are synthesized as precursors, and cleavage of both a signal peptide and acidic protein are required to yield the mature peptide (*53*, *86*).

Experimental data indicate accelerated evolution of the thionin mature domain under continuous selective pressure from pathogenic microorganisms that is consistent with its role in the innate immune system in plants. Bohlman and co-workers estimated between 50 and 100 copies of genes encoding leaf-specific thionins in barley (*34*). Later, nearly fifty variants of αHTH were identified in the *H. vulgare* genome with BLAST E-values ranging from 2 e-20 to 2 e-9, corresponding to 100% to 66% homology, respectively (*87*). Phylogenetic analysis shows a larger homology between leaf-specific thionins of dicots and monocots than between leaf- and seed-specific thionins of a same species, e.g. barley (*9*). Furthermore, comparison of the αHTH precursor with that of a neutral thionin (type V), which is quite divergent from other thionins, demonstrated a nearly 20% decreased homology in the mature thionin domain as compared to all other domains of the precursor (signal peptide, two introns, and acidic protein) (*88*). Ratios of non-synonymous (or changing physiochemical properties) to synonymous nucleotide substitution rates were considerably higher for the mature thionin than for signal peptide or acidic protein.

Four conserved motifs of the mature αHTH domain KSCCR/K, A/GRNCYNxCR, CRCK, F/YPK, and four disulfide bonds were found in 92 % of the identified homologues in the *H. vulgare* genome. These motifs include residues of the phospholipid-binding site and the R30 regulatory network (*60*). Thionin variants with mutations in the conserved motifs could have impaired activity.

Multiple alignment of the precursor of seed- and leaf-specific thionins with four disulfide bonds showed that the majority of thionins preserve the above conserved motifs in the mature thionin domain (Fig. 5) (*87*). Conservation in the acidic protein is considerably lower. In contrast, the C-terminal motif of signal peptide EQVQEG is highly conserved and carries two acidic and two polar residues. Interestingly, the N-terminus of the thionin signal peptide substantially varies among the seed- and leaf-specific thionins. Leaf-specific thionins possess an extended N-terminus while seed-specific peptides contain a second methionine residue. Translation initiation from the latter would produce an 18-residue-long signal peptide without basic residues at the N-terminus.

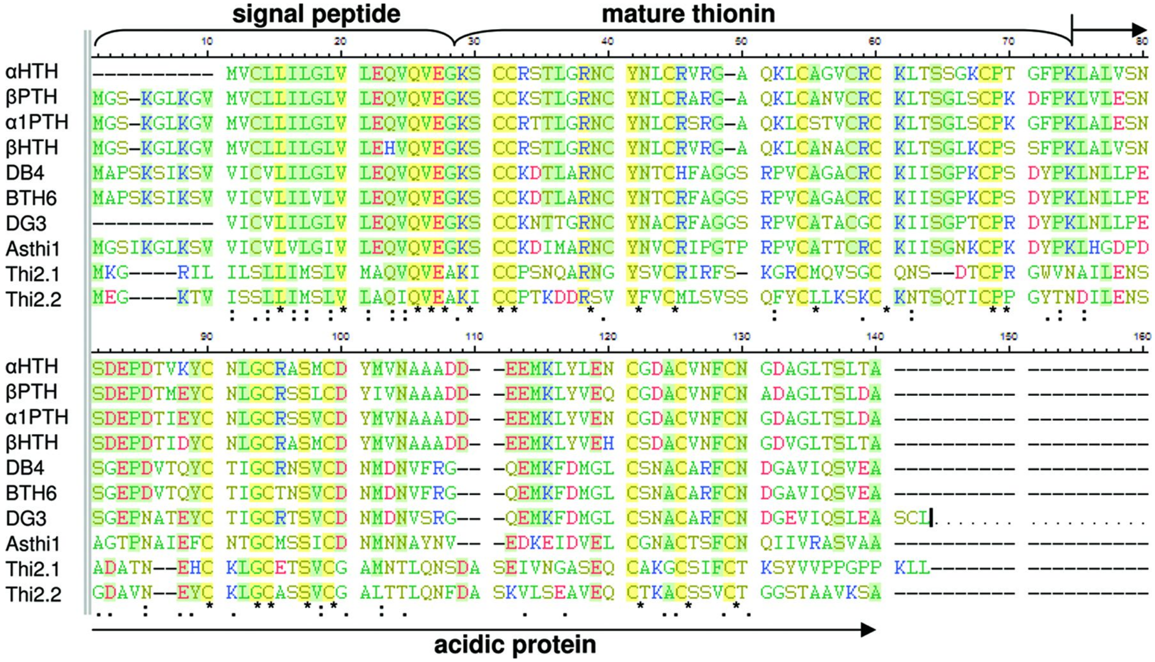
Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Function of Thionins in Plant Tissues

While plant cells produce and accumulate highly lytic thionins in concentrations that can be lethal for various microbial pathogens, the plant cells remain undamaged. The mechanism of such differential toxicity is not understood (*8*, *89*). *In situ*, the plant plasmalemma, as well as bacterial or fungal membranes, can be permeabilized by thionins (*90*). An acidic protein may help to neutralize the basic thionin in the precursor. However, after a mature thionin is cleaved from an acidic protein and a signal peptide, it should be prevented from penetrating and damaging plant membranes. Targeting and localization should play a significant role in protection of plant cells. Seed-specific thionins puro- and hordothionins accumulate in seed endosperm cells in high amounts and are deposited on the periphery of protein body membranes (*91*, *92*).

In contrast, leaf-specific thionins DB4 and BTH6 accumulate in cell walls of barley leaves (*34*, *49*) or intracellularly (*54*). Thionins were evenly distributed within cell walls of all leaf cells in etiolated seedlings, except for the outer cell wall of the epidermal cells, which contained increased amounts of thionin (*93*). Intracellular thionins were found in the central vacuole and were immunologically distinct from the cell-wall-bound thionins. Distribution of thionins was similar in four-week-old plants, albeit at considerably lower concentrations than in etiolated seedlings. High amounts of thionins were found in freshly formed cell-wall appositions at penetration sites in response to fungal infection (*49*). High levels of cell-wall-bound thionins in the infected regions of leaves were observed only in resistant barley cultivars. Another leaf-specific thionin from barley, DG3, was found predominantly in cell vacuoles and less than 1% in the cell-wall fraction (*86*). An extended acidic protein appears responsible for targeting the thionin DG3 to vacuoles, and the signal peptide remains fused to the mature vacuolar thionin. This could explain vacuolar accumulation without permeabilizing the tonoplast and damaging cells.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020



```
αHTH    ---------- MVCLLILGLV LEQVQVEGKS CCRSTLGRNC YNLCRVRG-A QKLCAGVCRC KLTSSGKCPT GFPKLALVSN
βPTH    MGS-KGLKGV MVCLLILGLV LEQVQVEGKS CCKSTLGRNC YNLCRARG-A QKLCANVCRC KLTSGLSCPK DFPKLVLESN
α1PTH   MGS-KGLKGV MVCLLILGLV LEQVQVEGKS CCRTTLGRNC YNLCRSRG-A QKLCSTVCRC KLTSGLSCPK GFPKLALESN
βHTH    MGS-KGLKGV MVCLLILGLV LEHVQVEGKS CCRSTLGRNC YNLCRVRG-A QKLCANACRC KLTSGLKCPS SFPKLALVSN
DB4     MAPSKSIKSV VICVLILGLV LEQVQVEGKS CCKDTLARNC YNTCHFAGGS RPVCAGACRC KIISGPKCPS DYPKLNLLPE
BTH6    MAPSKSIKSV VICVLILVLV LEQVQVEGKS CCKDTLARNC YNTCRFAGGS RPVCAGACRC KIISGPKCPS DYPKLNLLPE
DG3     ---------- VICVLILGLV LEQVQVEGKS CCKNTTGRNC YNACRFAGGS RPVCATACGC KIISGPTCPR DYPKLNLLPE
Asthi1  MGSIKGLKSV VICVLVLGIV LEQVQVEGKS CCKDIMARNC YNVCRIPGTP RPVCATTCRC KIISGNKCPK DYPKLHGDPD
Thi2.1  MKG----RIL ILSLLIMSLV MAQVQVEAKI CCPSNQARNG YSVCRIRFS- KGRCMQVSGC QNS--DTCPR GWVNAILENS
Thi2.2  MEG----KTV ISSLLIMSLV LAQIQVEAKI CCPTKDDRSV YFVCMLSVSS QFYCLLKSKC KNTSQTICPP GYTNDILENS
                   : .:*:: :* : ::***.*  **     *.  *  *      :  *    .* :      **   .: :

αHTH    SDEPDTVKYC NLGCRASMCD YMVNAAADD- -EEMKLYLEN CGDACVNFCN GDAGLTSLTA ---------- ----------
βPTH    SDEPDTMEYC NLGCRSSLCD YIVNAAADD- -EEMKLYVEQ CGDACVNFCN ADAGLTSLDA ---------- ----------
α1PTH   SDEPDTIEYC NLGCRSSVCD YMVNAAADD- -EEMKLYVEN CGDACVNFCN GDAGLTSLDA ---------- ----------
βHTH    SDEPDTIDYC NLGCRASMCD YMVNAAADD- -EEMKLYVEH CSDACVNFCN GDVGLTSLTA ---------- ----------
DB4     SGEPDVTQYC TIGCRNSVCD NMDNVFRG-- -QEMKFDMGL CSNACARFCN DGAVIQSVEA ---------- ----------
BTH6    SGEPDVTQYC TIGCTNSVCD NMDNVFRG-- -QEMKFDMGL CSNACARFCN DGAVIQSVEA ---------- ----------
DG3     SGEPNATEYC TIGCRTSVCD NMDNVSRG-- -QEMKFDMGL CSNACARFCN DGEVIQSLEA SCL....... ..........
Asthi1  AGTPNAIEFC NTGCMSSICD NMNNAYNV-- -EDKEIDVEL CGNACTSFCN QIIVRASVAA ---------- ----------
Thi2.1  ADATN--EHC KLGCETSVCG AMNTLQNSDA SEIVNGASEQ CAKGCSIFCT KSYVVPPGPP KLL------- ----------
Thi2.2  GDAVN--EYC KLGCASSVCG ALTTLQNFDA SKVLSEAVEQ CTKACSSVCT GGSTAAVKSA ---------- ----------
        ..  :  ..* . **  *:*.  : .        .  .    * ..*  .*.
```

Figure 5. Multiple alignments of selected precursors of seed- and leaf-specific thionins. (see color insert)

A signal peptide could protect a plant cell from the lytic activity of a thionin. Signal peptides of the barley leaf-specific cell-wall-bound thionins BD4 and BTH6 contain 27 amino acid residues. Meanwhile, secreting signal peptides as, for example, in the genes encoding the rice endochitinase or the Arabidopsis basic chitinase, can be 18-21-residue-long. In contrast, the well studied calreticulin apoplast targeting signal peptide, which delivers a cargo protein past the plasmalemma into the apoplast, consists of 29 residues (*94*). Chemokines, an essential part of the mammalian innate immune system, are expressed in a precursor form bearing a 32-34-residue signal peptide (*95*). Processing of these signal peptides results in different N-terminally truncated derivatives. Minor modifications of the N-terminus of chemokines may result in significantly enhanced or reduced biological activity or altered targeting. Some chemokines undergo up to five stepwise proteolytic truncations accompanied by an increase in biological activity. Similarly, extended signal peptides of the leaf-specific thionins could undergo stepwise processing to control membrane permeabilization activity and subsequent cell toxicity during targeting to a safe destination such as the cell wall.

Binding properties may play an important role in accumulation of thionins in high concentrations and in penetration of the fungal cells. Binding to plant cell wall components may keep thionins from inserting into the plasmalemma after the signal peptide is cleaved off and the phospholipid-binding site is revealed. Thionins contain up to 10 positively charged residues that can electrostatically interact with carboxyl groups of pectin and xylan (*68*). Various β-glucans and xylans of plant and bacterial origin bind to α1-purothionin, but cellulose and starch do not (*69*). β-Glucans comprise nearly 10% of the primary wall in monocots and are also found in bacterial and fungal cell walls. Furthermore, over 10 μg of α1-purothionin was bound to 2 μg of chitin, the main component of the fungal cell wall (*69*). Thus, thionin can bind to components of primary and secondary plant cell walls, as well as to components of bacterial and fungal cell walls. Binding to the bacterial or fungal cell wall brings thionin to the microbial plasmalemma.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Potential Role of Synergistic Enhancement in Thionin-Based Antifungal Protection

Although thionins exhibit high levels of antifungal activity *in vitro*, studies of natural thionin-based resistance and thionin overexpression suggest that thionins work in synergy with other antimicrobial proteins to reach full antifungal protection *in planta*. For example, exogenous overexpression of αHTH in Arabidopsis Col-0 rendered up to 60% antifungal resistance, while the resistant mutant UK-4 showed 80% resistance and the untransformed Col-0 had only 20% uninfected plants (*87*). The best Arabidopsis lines overexpressing the endogenous thionin *Thi2.1* gene showed only 70% of the antifungal resistance shown by the resistant mutant UK-4 (*37*). Similar results were obtained when this gene was overexpressed in tomato (*50*). However, transcription levels of the transgenic *Thi2.1* as well as *hth1* encoding αHTH greatly exceeded levels of *Thi2.1* in UK-4. as compared to in UK-4. The UK-4 mutant showed only a five- to ten-fold increase in the *Thi2.1* transcription level as compared to the endogenous level

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

and, in addition, the mutant overexpressed at least three other proteins (*39*). No correlations of other genes with antifungal activity have been found so far. Synergistic interactions of Thi2.1 with the other protein(s) in UK-4 could explain these results.

Studies of a thionin-based resistance in the resistant barley cultivars also suggest the presence of cooperative action of thionins with other defense proteins (*49*). Pathogenic fungi release a variety of proteins and carbohydrates during invasion, among which different types of proteases have been identified (*96–98*). Plants in response produce protease inhibitors (*99*, *100*). Antifungal activity of wheat and barley thionins is synergistically enhanced *in vitro* by barley trypsin inhibitors (*35*). The same study demonstrated that two seed storage proteins, 2S albumins from radish and oilseed rape, increased fungal inhibition activity of thionins up to 73-fold when added into the culture medium at noninhibitory concentrations. Both 2S albumins display a weak lytic activity. These data indicate at least two classes of protein candidates for complementing thionin-based plant protection.

Transgenic Expression of Thionin

While transgenic overexpression of leaf-specific thionins was successfully reported by several groups of scientists, exogenous expression of seed thionins has produced variable results. However, recent advances in understanding post-translational expression of thionin precursors make the idea of using seed-specific thionin for crop protection more realistic.

Among leaf-specific thionins, successful overexpression was reported for a thionin Thi2.1 from Arabidopsis and a cell-wall-bound thionin Asthi1 from oat. Endogenous overexpression of Thi2.1 enhanced plant resistance to *Fusarium oxysporum* (*37*) (Table II). In that work, Thi2.1 was detected in total protein extracts of seedlings by immunoblot analysis. Exogenous expression of Thi2.1 in tomato enhanced resistance to bacterial wilt and Fusarium wilt. The transgenic oat leaf-specific thionin Asthi1 accumulated in cell walls when expressed in rice, similarly to the barley leaf-specific thionins (*51*). Overexpression of thionin did not affect expression levels of other defense-related proteins PR-1 and PR-5 in transgenic plants (*37*).

Among seed-specific thionins, experiments on transgenic expression were reported for α1-purothionin, βPTH, αHTH, and β-hordothionin (*101–103*, *105*). The results of overexpression in leaf tissues were inconsistent, despite the use of promoters that were functional in leaves. The transgenic αHTH accumulated in seeds, but not in leaves of transgenic oat when expressed under the wild-type signal peptide with the acidic protein (*103*). The maize *ubi1* promoter, which is active in leaf tissues, and the αHTH cDNA were used in that work. The purified transgenic αHTH from oat seed completely inhibited growth of *Fusarium graminearum* at 3 μM, a concentration observed in previous work (*35*, *42*). In the earlier work, the genomic DNA encoding the wild-type αHTH precursor was successfully expressed in tobacco leaves, while expression of α1-purothionin from the cDNA produced significantly lower amounts of thionin (*101*). The transgenic αHTH accumulated in tobacco leaf tissues if its cDNA was combined with the signal peptide from barley leaf thionin either with or without the acidic protein, but not in the absence of a signal peptide (*102*). Meanwhile, expression of β-hordothionin under the same signal peptide failed. No *in planta* increase in antibacterial activity was found in transgenic tobacco lines expressing αHTH (*102*). However, an *in vitro* inhibition assay showed similar inhibitory activity of the HPCL-purified transgenic αHTH with that of the wild-type αHTH.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Table II. Transgenic expression of thionins *in planta*

Peptide	*Origin*	*Transgenic expression*			
		Transformed Plant	*Signal peptide*	*Localization in leaf tissues*	*References*
α1-PTH	Wheat seed	Tobacco	Native	Not detected	(*101*)
β-HTH	Barley seed	Tobacco	None	Not detected	(*102*)
		Tobacco	Barley leaf DB4	Not detected	(*102*)
α-HTH	Barley seed	Tobacco*	Native	50 mM H_2SO_4 extract	(*101*)
		Tobacco	None	Not detected	(*102*)
		Tobacco	Barley leaf DB4	Membrane, microsome	(*102*)
		Oat	Native	Not detected †	(*103*)
		Apple*	Barley leaf DB4	Not detected	(*104*)
β-PHT	Wheat seed	Arabidopsis *	Rice endochitinase	Total protein extract	(*105*)
Asthi1	Oat leaf	Rice *	Native	Total protein, cell wall	(*51*)
Thi2.1	Arabidopsis leaf	Arabidopsis	Native	Total protein	(*37*)
		Tomato *	Native	Not determined	(*37*)

* Enhanced antibacterial resistance was obtained in transgenic plants; † detected in seed.

Thionin Renders the Highest Antimicrobial Resistance *in Planta*

The first successful improvement of antifungal resistance *in planta* upon overexpression of a seed-specific thionin was reported for βPTH in Arabidopsis (*105*). βPTH was expressed under an secreting signal peptide together with the acidic protein using a cDNA of the *purA* gene. In that work, βPTH was directly compared with two linear amphipathic antimicrobial peptides, cecropin

B and phor21 to verify antifungal activity *in vitro* (*42*). Cecropin B is a natural antimicrobial peptide from *Cecropia* moth, and phor21 is a synthetic antimicrobial peptide. All three peptides showed relatively high antifungal activity *in vitro* (Table I). The peptides were expressed under an endogenous promoter with a moderate-level of transcriptional activity; the *A. thaliana* chloroplast carbonic anhydrase promoter; and the rice endochitinase signal peptide for extracellular excretion of transgenic peptides. Two homozygous lines with the highest levels of transgene expression, which were obtained after screening 24 to 30 independent transformation events per construct, were tested in the antibacterial (*P. syringae*) together with the antifungal bioassays (*F. oxysporum*). Expression of βPTH rendered the greatest antibacterial and antifungal resistance, while cecropin B enhanced only antibacterial activity and phor21 did not improve antimicrobial resistance. The transgenic βPTH arrested fungal growth on leaf surfaces and prevented infection of stomata. Growth anomalies of the fungal hyphae on leaves of the transgenic lines were similar to those observed in Arabidopsis overexpressing thionin Thi2.1 and fungicides (*37*, *106*). The *in planta* antimicrobial activities of the tested peptides were consistent with previously reported *in vitro* results when considering together the levels of growth inhibition activity and the levels of resistance to proteolytic degradation (Fig. 6). Interestingly, substitution of the native signal peptide in the βPTH precursor with a conventional secretion signal peptide did not interfere with production of an active thionin.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Expression of αHTH without the acidic protein considerably lowered accumulation of the transgenic αHTH in tobacco leaves (*102*). Modification of the βPTH precursor by fusing a green fluorescence protein (EGFP) to a carboxyl-end of the acidic protein impaired antimicrobial activity *in planta* even though EGFP was post-translationally cleaved (*105*). An acidic protein was thought to neutralize the highly basic mature peptide inside a plant cell (*54*). However, decreased accumulation and activity of the mature thionin could result from partial misfolding. Secondary structure of seed thionins contains four highly conserved disulfide bonds. These disulfide bonds introduce a major constraint on the tertiary structure of the 45 residue-long peptide, especially around R30 (*59*, *78*). The acidic protein may facilitate correct folding of the mature peptide, and fusion of EGFP could interfere with this function, lowering activity of βPTH (*105*).

Six cysteine residues are highly conserved in the acidic protein, which could explain the observed sensitivity of thionin to modification of the C-terminus (*87*). A homology analysis of the αHTH precursor showed a significantly lower conservation in the acidic protein as compared to the mature domain and the signal peptide (Fig. 5). Unlike the acidic protein, the majority of the thionin signal peptide domain is conserved within the 4DSB subfamily. Conservation of the C-terminal motif EQVQEG persists to the 3DSB subfamily, becoming just one amino acid residue shorter and with no functionally significant substitutions. In contrast, substantial differences are found between the N-termini of signal peptides of the seed- and the leaf-specific thionins of the 4DSB subfamily.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Signal Peptide Affects Thionin Antimicrobial Activity and Plant Viability

The homology analysis of the αHTH precursor implied a complex function of the thionin signal peptide. This evidence prompted us to investigate effects of the signal peptide sequence on thionin expression *in planta*. The signal peptide encoding sequence of the cDNA of the wild-type αHTH precursor was replaced with three different signal peptides, and the chimeric precursors were expressed in Arabidopsis under a strong constitutive promoter (*87*). The signal peptide directly connects to K1, which belongs to the phospholipid-binding site (*55*, *56*). The negatively charged C-terminal motif of the thionin signal peptide may electrostatically interact with the positively charged phospholipid-binding site, blocking lytic activity. To test this hypothesis, the secreting signal peptide from the rice glycine-rich protein (SPA) was inserted upstream of the αHTH mature peptide. This signal peptide, as cloned in pCAMBIA 1305.2, leaves 6 extra amino acid residues at the N-terminus of a recombinant protein. The second signal peptide SPB was designed to test the extended N-terminus in signal peptides of leaf-specific thionins. The N-terminal sequence from the leaf-specific oat thionin Asthi1 was fused to the wild-type signal peptide of αHTH to create the 28-residue-long hybrid signal peptide SPB. The third signal peptide, the secreting signal peptide of the Arabidopsis basic chitinase (SPC) with a well-documented excretion function, represented a conventional secreting signal peptide.

Analysis of the selected homozygous lines revealed that the C-terminal motif of the thionin signal peptide protects plant cells from lytic activity of thionin (*87*). Expression of αHTH under the conventional secreting signal peptide SPC affected plant viability because only lines with low levels of transgene expression were selected for the SPC-αHTH fusion despite additional rounds of screening (Table III, SPC). Meanwhile, addition of the extra six residues, which resemble the conserved C-terminal motif of the thionin signal peptide, at the N-terminus of the mature thionin in case of the SPA-αHTH fusion removed the thionin toxicity because several lines with relatively high levels of transgene expression were identified. Moreover, thionin expressed under SPA became inactive because antifungal resistance remained at the level of untransformed plants. This fact suggests that the extra residues blocked the phospholipid-binding site rendering the chimeric thionin inactive.

Expression of αHTH under the hybrid signal peptide SPB, which carried the native C-terminal motif and the N-terminal motif of the thionin leaf-specific signal peptide, did not affect plant viability and produced an active αHTH (Table III, SPB). The selected homozygous lines displayed enhanced resistance to *F. oxysporum* up to 60%. This is a substantial improvement from 20% found for untransformed plants that was achieved by overexpression of a single gene as compared to the resistant mutant UK4 with several upregulated genes. The signal peptide SPC also enhanced antifungal resistance, confirming that the conserved C-terminal motif is not involved in thionin folding. Thus, secretion of biologically active αHTH outside the plasmalemma under an exogenous signal sequence can impair plant viability. This fact suggests that the wild-type signal peptide has an additional function(s) besides targeting the mature peptide.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

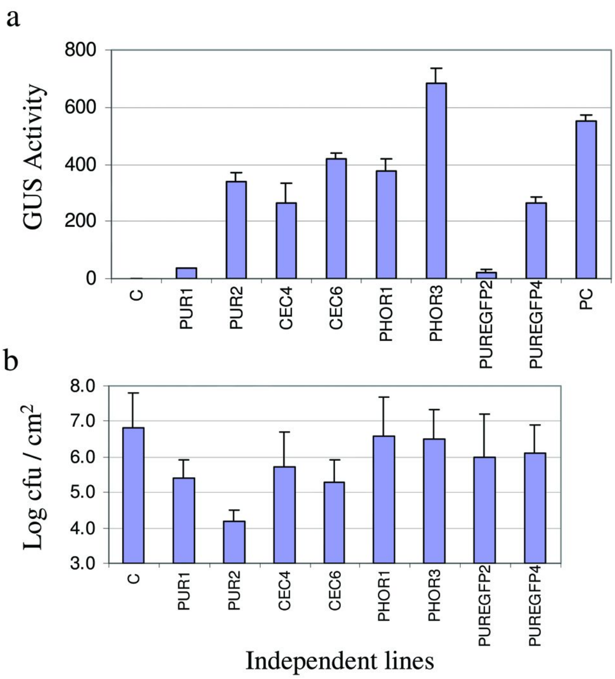


Figure 6. Levels of transgene expression and antibacterial resistance in transgenic Arabidopsis lines expressing βPTH (PUR), cecropin B (CECB), and phor21 (PHOR) (from (105)). (a) Relative levels of expression were measured as β-glucuronidase (GUS) activity in pmol MU per mg of protein per min (2 experiments x 6 reps). (b) in planta antibacterial assay. Growth inhibition of Pseudomonas syringae pv tomato DC3000 was measured in leaf tissues of 5-weeks-old plants 4 days after inoculation (2 experiments x 5 reps). C, untransformed Col-0 (negative control); PC, a homozygous line with a high level of GUS activity (positive control).

To further investigate effects of the signal peptide sequences on stability, localization, and activity of transgenic thionins, the αHTH precursor variants with the signal peptides SPA, SPB, and SPC were transiently expressed in *Nicotiana benthamiana* (Oard, unpublished results). MS analysis of the major HPLC fractions revealed that only SPB released the correctly processed mature peptide, with a molecular weight corresponding to 45 amino acid residues. By contrast, the main product for SPC carried one extra residue at the N-terminus indicating incorrect processing. A minor, 43-residue peak for SPC pointed to reduced stability. SPA released a 47-residue peptide with two extra residues at the N-terminus instead of the expected six residues. Nonetheless, these two extra residues, one of which was negatively charged, were sufficient to block the lytic activity of thionin (Table III). MS analysis confirmed that the additional fractions for SPB contained two, three, and ten extra residues at the N-terminus of the mature thionin, suggesting step-wise processing of the thionin signal peptide similar to that of chemokines (*95*).

Table III. Arabidopsis transgenic lines expressing αHTH under different signal peptides

Signal peptide	*№ of selected T_0 plants* ‡	*The highest relative activity**		*% of infected plants in the best T_2 line*†
		T_0 plants	*T_2 lines*	
SPA	49	510	340 (±20)	80-90
SPB	50	420	810 (±15)	40-50
SPC	65	15	20(±3)	40-50
Col-0	-	0	0	80-90
UK-4	-	ND	ND	20-30

‡ Plants with normal phenotype. * Relative activity was measured as described (*105*). Results for T_2 homozygous lines represent avg ± std of 6-8 plants x 3 rep. calculated per mg of fresh leaf tissue. † Antifungal resistance bioassay performed as described (*37*); 3 exp x 3 replicates.

Evidence for the critical role of signal peptide for expression of thionins in leaf tissues is consistent with experimental data for the linear antimicrobial peptide cecropin B (*107*). Expression of the latter under a conventional secreting signal peptide of rice chitinase increased antimicrobial activity of transgenic rice as compared to the native signal peptide. Oard and co-workers showed that simply secreting an antimicrobial peptide outside the plasmalemma can damage plant cells and impair plant viability (*87*). The C-terminal motif of the thionin signal peptide protects a plant cell while thionin is inside the cell. Questions about what prevents thionin from reentering plasmalemma and how thionins become integrated into the plant cell wall remain unanswered. The extended positively charged N-terminal motif of the leaf-specific thionin signal peptide could facilitate binding to the negatively charged plant cell wall.

Recently, the durability of resistance introduced by thionins was demonstrated in apple (*104*). The engineered gene encoding αHTH under the signal peptide from a leaf-specific thionin was transformed into two apple cultivars. Six transgenic lines were tested in a field trial for four consecutive years after inoculation with a fungal pathogen *Verturia inaequalis*. Four out of six lines were significantly less susceptible to apple scab during the entire four-year period. PCR analysis confirmed the presence of the transgene in 12-year-old trees. Interestingly, no thionin was detectable in apple leaf extracts of any resistant line although proteins were extracted with 50 mM H_2SO_4. Low expression levels could be due to suboptimal signal peptide. These data underline the importance of studying functions of thionin signal peptide to design an optimized sequence to achieve desirable levels of thionin accumulation in leaf tissues.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Conclusion

The discovery of a complex regulatory function of the thionin signal peptide lays a foundation for developing thionin-based strategies for crop protection. Alone or in combination with enhancers, potent seed-specific thionins from food crops have the potential to protect foliar tissues against a wide range of bacterial and fungal diseases. Such a strategy significantly reduces or may even eliminate safety concerns associated with genetically engineered organisms. To date, thionins present no interference with other plant defense signal pathways. Moreover, the chance to overcome the disease resistance of thionin-expressing crops by naturally occurring thionin-resistant pathogens would be considerably desceased because phosphatidylserine, the primary target of thionin, is highly conserved in microbial membranes.

Seed-specific thionins demonstrate potentially excellent characteristics as candidates for molecular breeding of crops with improved antimicrobial resistance: broad range of antimicrobial activity, low lethal concentrations, and rapid mode of action. Until very recently, thionins were considered toxic for human and animal consumption and therefore not suitable for molecular breeding. βPTH and αHTH have withstood the test of time with several centuries of safe consumption by humans and animals. Thionins, therefore, hold considerable promise for developing a broad-rage antimicrobial defense system for crop protection.

The most recent work with thionin precursors indicates evidence of intricate post-translational regulation of antimicrobial thionins in plant cells, providing a framework for investigation of mechanisms evolved by plants to fine tune their innate immune systems. The results to date vastly improve our understanding and the efficacy of thionin-based defense, but also demonstrate the need for further work, as complete pathogen resistance of transgenic plants has yet to be obtained, and only a limited number of hosts and plant pathogens have been investigated. Understanding of these mechanisms will enable us to manipulate these immune agents to extend protection to economically important plant species.

References

1. Bourne, J. J. *National Geographic* **2009** (June issue).
2. Agrios, G. *Plant Pathology*, 4th ed.; Academic Press: London, U.K., 1997.
3. Gust, A.; Brunner, F.; Nürnberger, T. *Curr. Opin. Biotechnol.* **2010**, *21*, 204–10.
4. Kawata, M.; Nakajima, T.; Yamamoto, T.; Mori, K.; Oikawa, T.; Fukumoto, F.; Kuroda, S. *Jpn. Agric. Res. Q.* **2003**, *37*, 71–76.
5. Hancock, R.; Lehrer, R. *Trends Biotechnol.* **1998**, *16*, 82–88.
6. Veronese, P.; Ruiz, M.; Coca, M.; Hernandez-Lopez, A.; Lee, H.; Ibeas, J.; Damsz, B.; Pardo, J.; Hasegawa, P.; Bressan, R.; Narasimhan, M. *Plant Physiol.* **2003**, *131*, 1580–90.
7. Zasloff, M. *Nature* **2002**, *415*, 389–395.
8. Florack, D.; Stiekema, W. *Plant Mol. Biol.* **1994**, *26*, 25–37.

9. García-Olmedo, F.; Rodriguez-Palenzuela, P.; Hernandez-Lucas, C.; Ponz, F.; Maraña, C.; Carmona, M.; Lopez-Fando, J.; Fernandez, J.; Carbonero, P. *Oxford Surve. Plant Mol. Cell Biol.* **1989**, *6*, 31–60.
10. Stec, B. *Cell Mol. Life Sci.* **2006**, *63*, 1370–85.
11. Agrios, G. N. *Plant Pathology*, 5th ed.; Elsevier Academic Press: New York, 2005.
12. Glazebrook, J. *Annu. Rev. Phytopathol.* **2005**, *43*, 205–227.
13. Smith, J. L.; De Moraes, C. M.; Mescher, M. C. *Pest Manag. Sci.* **2009**, *65*, 497–503.
14. Bernoux, M.; Ellis, J. G.; Dodds, P. N. *Curr. Opin. Plant Biol.* **2011**, *14.*
15. Jones, D. G.; Dangl, J. L. *Nature* **2006**, *444.*
16. Dodds, P. N.; Rathjen, J. P. *Nat. Rev. Genet.* **2010**, *11*, 539–548.
17. Chisholm, S. T.; Coaker, G.; Day, B.; Staskawicz, B. J. *Cell* **2006**, *124*, 803–814.
18. Fellbrich, G.; Romanski, A.; Varet, A.; Blume, B.; Brunner, F.; Engelhardt, S.; Felix, G.; Kemmerling, B.; Krzymowska, M.; Nürnberger, T. *Plant J.* **2002**, *32*, 375–390.
19. Walter, S.; Nicholson, P.; Doohan, F. *New. Phytol.* **2010**, *185*, 54–66.
20. Metraux, J.; Jackson, R.; Schnettler, E.; Goldbach, R. In *Plant Innate Immunity*; Academic Press: New York, 2009; Vol. 51, pp 39−89
21. Collinge, D.; Jørgensen, H.; Lund, O.; Lyngkjaer, M. *Annu. Rev. Phytopathol.* **2010**, *48*, 269–91.
22. Shewry, P.; Jones, H.; Halford, N. In *Food Biotechnology*; Springer: New York, 2008; Vol. 111, pp 149−186.
23. Brigulla, M.; Wackernagel, W. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1027–41.
24. Manimaran, P.; Ramkumar, G.; Sakthivel, K.; Sundaram, R.; Madhav, M.; Balachandran, S. *Biotechnol. Adv.* 2011, Epub ahead of print.
25. Mackey, D.; Bent, A. F. *Annu. Rev. Phytopathol.* **2007**, *45*, 399–436.
26. Dathe, M.; Wieprecht, T. *Biochem. Biophys. Acta* **1999**, *1462*, 71–87.
27. Aerts, A.; François, I.; Cammue, B.; Thevissen, K. *Cell Mol. Life Sci.* **2008**, *65*, 2069–79.
28. Bechinger, B. *Crit. Rev. Plant Sci.* **2004**, *23*, 271–292.
29. Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449–53.
30. Powell, W.; Catranis, C.; Maynard, C. *Mol. Plant-Microbe Interact.* **1995**, *8*, 792–794.
31. Rajasekaran, K.; Stromberg, K.; Cary, J.; Cleveland, T. *J. Agric. Food Chem.* **2001**, *49*, 2799–2803.
32. Alan, A. R.; Blowers, A.; Earle, E. D. *Plant Cell Rep.* **2004**, *22*, 388–396.
33. Alan, A. R.; Earle, E. D. *Mol. Plant-Microbe Interact.* **2002**, *15*, 701–708.
34. Bohlmann, H.; Clausen, S.; Behnke, S.; Giese, H.; Hiller, C.; Reimann-Philipp, U.; Schrader, G.; Barkholt, V.; Apel, K. *EMBO J.* **1988**, *7*, 1559–1565.
35. Terras, F.; Schoofs', H.; Thevissen, K.; Osborn, R.; Vanderleyden, J.; Cammue, B.; Broekaert, W. F. *Plant Physiol.* **1993**, *103*, 1311–1319.
36. Garcia-Olmedo, F.; Molina, A.; Alamillo, J. M.; Rodriguez-Palenzuela, P. *Biopolymers* **1998**, *47*, 479–91.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

37. Epple, P.; Apel, K.; Bohlmann, H. *Plant Cell* **1997**, *4*, 509–20.
38. Sels, J.; Mathys, J.; De Coninck, B.; Cammue, B.; De Bolle, M. *Plant Physiol. Biochem.* **2008**, *46*, 941–50.
39. Epple, P.; Vignutelli, A.; Apel, K.; Bohlmann, H. *Mol. Plant-Microbe Interact.* **1998**, *11*, 523–9.
40. Kitanaga, Y.; Jian, C.; Hasegawa, M.; Yazaki, J.; Kishimoto, N.; Kikuchi, S.; Nakamura, H.; Ichikawa, H.; Asami, T.; Yoshida, S.; Yamaguchi, I.; Suzuki, Y. *Biosci. Biotech. Biochem.* **2006**, *70*, 2410–9.
41. Thevissen, K.; Terras, F.; Broekaert, W. F. *Appl. Env. Microbiol.* **1999**, *63*, 5451–5458.
42. Oard, S.; Rush, M. C.; Oard, J. H. *J. Appl. Microbiol.* **2004**, *97*, 169–80.
43. Niidome, T.; Anzai, S. *J. Pept. Sci.* **1999**, *5*, 298–305.
44. Richard, J.; Kelly, I.; Marion, D.; Auger, M.; Pézolet, M. *Biochemistry* **2005**, *44*, 52–61.
45. Huang, W.; Vernon, L. P.; Hansen, L. D.; Bell, J. D. *Biochemistry* **1997**, *36*, 2860–6.
46. Bechinger, B. *Cel. Mol. Life Sci.* **2008**, *65*, 3028–39.
47. Scheller, A.; Oehlke, J.; Wiesner, B.; Dathe, M.; Krause, E.; Beyermann, M.; Melzig, M.; Bienert, M. *J. Pept. Sci.* **1999**, *5*, 185–194.
48. Thevissen, D.; Ghazi, A.; De Samblanx, G. W.; Brownlee, C.; Osborn, R. W.; Broekaert, W. F. *J. Biol. Chem.* **1996**, *271*, 15018–15025.
49. Ebrahim-Nesbat, F.; Behnke, S.; Kleinhofs, A.; Apel, K. *Planta* **1989**, *179*, 203–210.
50. Chan, Y.; Prasad, V.; Chen, K.; Liu, P.; Chan, M.; Cheng, C. *Planta* **2005**, *221*, 386–393.
51. Iwai, T.; Kaku, H.; Honkura, R.; Nakamura, S.; Ochiai, H.; Sasaki, T.; Ohashi, Y. *Mol. Plant-Microbe Interact.* **2002**, *15*, 515–521.
52. Rayapuram, C.; Wu, J.; Haas, C.; Baldwin, I. *Mol. Plant-Microbe Interact.* **2008**, *21*, 988–1000.
53. Van Campenhout, S.; Sagi, L.; Vander Stappen, J.; Vlockaert, G. *Theor. Appl. Genet.* **1998**, *96*, 80–86.
54. Reimann-Philipp, U.; Schrader, G.; Martinoia, E.; Barkholt, V.; Apel, K. *J. Biol. Chem.* **1989**, *264*, 8978–84.
55. Rao, U.; Stec, B.; Teeter, M. *Acta Crystallog., Sect. D* **1995**, *D51*, 904–913.
56. Stec, B.; Rao, U.; Teeter, M. M. *Acta Crystallogr., Sect. D* **1995**, *51*, 914–924.
57. Stec, B.; Markman, O.; Rao, U.; Heffron, G.; Henderson, S.; Vernon, L. P.; Brumfeld, V.; Teeter, M. M. *J. Pept. Res.* **2004**, *64*, 210–224.
58. Oard, S.; Enright, F.; Li, B. *Biophys. Chem.* **2010**, *147*, 42–52.
59. Oard, S.; Karki, B.; Enright, F. *Biophys. Chem.* **2007**, *130*, 65–75.
60. Oard, S. *Biochem. Biophys. Acta* **2011**, *1808*, 1737–45.
61. Bekes, F.; Lasztity, R. *J. Cereal Chem.* **1981**, *58*, 360–361.
62. Fujimura, M.; Ideguchi, M.; Minami, Y.; Watanabe, K.; Tadera, K. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 571–577.
63. Vernon, L.; Evett, G. E.; Zeikus, R. D.; Gray, W. R. *Arch. Biochem Biophys* **1985**, *238*, 18–29.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

64. Hughes, P.; Dennis, E.; Whitecross, M.; Llewellyn, D.; Gage, P. *J. Biol. Chem.* **2000**, *275*, 823–927.

65. Evans, J.; Wang, Y.; Shaw, K.; Vernon, L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5849–53.

66. Coulson, E. J.; Harris, T. H.; Axelrod, B. *Cereal Chem.* **1942**, *19*, 301–307.

67. Kramer, K. J.; Klassen, L. W.; Jones, B. L.; Speirs, R. D.; Kammer, A. E. *Toxicol. Appl. Pharmacol.* **1979**, *48*, 179–83.

68. Brett, C.; Waldron, K. *Physiology and Biochemistry of Plant Cell Walls*, 2nd ed.; Chapman and Hall: London, 1996.

69. Oita, S.; Ohnishi-Kameyama, M.; Nagata, T. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 958–64.

70. Bohlmann, H. *Crit. Rev. Plant Sci.* **1994**, *13*, 1–16.

71. Caaveiro, J.; Molina, A.; Rodriguez-Palenzuela, P.; Goni, F.; Gonzalez-Manas, J. *Protein Sci.* **1998**, *7*, 2567–2577.

72. Bechinger, B.; Lohner, K. *Biochim. Biophys. Acta.* **2006**, *1758*, 1529–1539.

73. Richard, J.-A.; Kelly, I.; Marion, D.; Pezolet, M.; Auger, M. *Biophys. J.* **2002**, *83*, 2074–2083.

74. Llanos, P.; Henriquez, M.; Minic, J.; Elmorjani, K.; Marion, D.; Riquelme, G.; Molgó, J.; Benoit, E. *FEBS J.* **2006**, *273*, 1710–22.

75. Vila-Perello, M.; Sanchez-Vallet, A.; Garcia-Olmedo, F.; Molina, A.; Andreu, D. *J. Biol. Chem.* **2005**, *280*, 1661–1668.

76. Fracki, W.; Li, D.; Owen, N.; Perry, C.; Naisbitt, G.; Vernon, L. *Toxicon* **1992**, *30*, 1427–1440.

77. Wada, K.; Ozaki, Y.; Matsubara, H.; Yoshizumi, H. *J. Biochem.* **1982**, *91*, 257–63.

78. Oard, S.; Karki, B. B. *Biophys. Chem.* **2006**, *121*, 30–43.

79. Gomes, D.; Agasse, A.; Thiébaud, P.; Delrot, S.; Gerós, H.; Chaumont, F. *Biochim. Biophys. Acta* **2009**, *1788*, 1213–28.

80. Gurtovenko, A.; Onike, O.; Anwar, J. *Langmuir* **2008**, *24*, 9656–60.

81. Van Etten, C. H.; Nielsen, H. C.; Peters, J. E. *Phytochemistry* **1965**, *4*, 467–473.

82. Epple, P.; Apel, K.; Bohlmann, H. *Plant Physiol.* **1995**, *109*, 813–820.

83. Xu, L.; Liu, F.; Wang, Z.; Peng, W.; Huang, R.; Huang, D.; Xie, D. *FEBS Lett.* **2001**, *494*, 161–164.

84. Valè, G.; Torrigiani, E.; Gatti, A.; Delogu, G.; Porta-Puglia, A.; Vannacci, G.; Cattivelli, L. *Physiol. Mol. Plant Pathol.* **1994**, *44*, 207–215.

85. Bohlmann, H.; Apel, K. *Mol. Gen. Genet.* **1987**, *207*, 446–454.

86. Romero, A.; Alamillo, J.; Garcia-Olmedo, F. *Eur. J. Biochem.* **1997**, *243*, 202–208.

87. Oard, S.; Enright, F. *Plant J.* **2011** submitted for publication.

88. Castagnaro, A.; Maraña, C.; Carbonero, P.; García-Olmedo, F. *J. Mol. Biol.* **1992**, *224*, 1003–9.

89. Brümmer, J.; Thole, H.; Kloppstech, K. *Eur. J. Biochem.* **1994**, *219*, 425–33.

90. Reimann-Philipp, U.; Behnke, S.; Batschauer, A.; Schäfer, E.; Apel, K. *Eur. J. Biochem.* **1989**, *182*, 283–289.

91. Carbonero, P.; Garcia-Olmedo, F.; Hernandez-Lucas, C. *J. Agric. Food Chem.* **1980**, *28*, 399–402.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

92. Ponz, F.; Paz-Ares, J.; Hernández-Lucas, C.; Carbonero, P.; García-Olmedo, F. *EMBO J.* **1983**, *2*, 1035–40.
93. Ebrahim-Nesbat, F.; Bohl, S.; Heitefuss, R.; Apel, K. *Physiol. Mol. Plant Pathol.* **1993**, *43*, 343–352.
94. Borisjuk, N.; Sitailo, L.; Adler, K.; Malysheva, L.; Tewes, A.; Borisjuk, L.; Manteuffel, R. *Planta* **1998**, *206*, 504–14.
95. Mortier, A.; Van Damme, J.; Proost, P. *Pharmacol. Therapeut.* **2008**, *120*, 197–217.
96. Valueva, T.; Mosolov, V. *Biochemistry (Mosc)* **2004**, *69*, 1305–9.
97. Pekkarinen, A.; Jones, B.; Niku-Paavola, M. *Eur. J. Biochem.* **2002**, *269*, 798–807.
98. Sreedhar, L.; Kobayashi, D.; Bunting, T.; Hillman, B.; Belanger, F. *Gene* **1999**, *235*, 121–9.
99. Pekkarinen, A.; Jones, B. *J. Agric. Food Chem.* **2003**, *51*, 1710–7.
100. Joshi, B. N.; Sainani, M.; Bastawade, K.; Gupta, V.; Ranjekar, P. K. *Biochem. Biophys. Res. Comm.* **1998**, *246*, 382–387.
101. Carmona, M.; Molina, A.; Fernández, J.; López-Fando, J.; García-Olmedo, F. *Plant J.* **1993**, *3*, 457–62.
102. Florack, D.; Dirkse, W.; Visser, B.; Heidekamp, F.; Stiekema, W. *Plant Mol. Biol.* **1994**, *24*, 83–96.
103. Carlson, A.; Skadsen, R.; Kaeppler, H. *In Vitro Cell. Dev. Biol.: Plant* **2006**, *42*, 318–323.
104. Krens, F.; Schaart, J.; Groenwold, R.; Walraven, A.; Hesselink, T.; Thissen, J. *Transgenic Res.* 2011, Epub ahead of print.
105. Oard, S.; Enright, F. *Plan Cell Rep.* **2006**, *25*, 561–572.
106. Robson, G.; Kuhn, P.; Trinci, A. *J. Gen. Microbiol.* **1988**, *134*.
107. Sharma, A.; Sharma, R.; Imamura, M.; Yamakawa, M.; Machii, H. *FEBS Lett.* **2000**, *484*, 7–11.

Downloaded by STANFORD UNIV GREEN LIBR on May 29, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch020

Chapter 21

Transgenic Expression of Antimicrobial Peptides in Plants: Strategies for Enhanced Disease Resistance, Improved Productivity, and Production of Therapeutics

Dmytro P. Yevtushenko[*] and Santosh Misra

Centre for Forest Biology, University of Victoria,
Victoria, British Columbia, V8W 3P6, Canada
[*]E-mail: dmytro@uvic.ca

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

Antimicrobial peptides represent a diverse group of small membrane-active molecules that are essential components of the innate defense system of, probably, all living organisms. In this chapter, we demonstrate that the transformation of plants with genes encoding for antimicrobial peptides have enabled the development of plants with enhanced and durable resistance to many diseases, increased crop yields, and reduced post-harvest losses. *In vitro*, the peptides inhibited plant-specific pathogens at micromolar concentrations that were not toxic to plant protoplasts or mammalian cells. The majority of the antimicrobial peptides retained their activities in heterologous plant systems. Most importantly, the expression level of antimicrobial peptides in transgenic plants, regulated by promoters with appropriate level of spatiotemporal activity, was sufficient to confer resistance against a variety of pathogenic fungi, oomycetes, and bacteria. The accumulation of antimicrobial peptides in transgenic plants did not alter normal plant growth or development, even in the lines with the highest level of transgene expression. These studies are greatly expanding our knowledge of the interactions between antimicrobial peptides, phytopathogens, and host plants. In turn, this is helping the development of novel strategies for sustainable agriculture, including the engineering of new crop varieties with broad-spectrum disease resistance that will

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

require fewer pesticide applications, reduce the associated environmental risks, and provide higher yields. In addition, the transgenic expression of antimicrobial peptides in plants is emerging as one of the most promising platforms for the cost-effective production of tomorrow's therapeutics.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

Introduction

The widespread distribution of antimicrobial peptides (AMPs) in phylogenetically distant organisms, ranging from bacteria and primitive invertebrates to humans, suggests that they are evolutionary ancient molecules (*1*), being used by their respective host organisms to fend off a wide range of pathogenic bacteria, fungi, protozoa (including *Trypanosoma brucei* that causes sleeping sickness), parasites, and even enveloped viruses like HIV and herpes simplex virus (*2*). Moreover, some of the peptides are effective against cancer cells (*3*, *4*). The most important and unique feature of AMPs, which gives them a well-defined advantage over other defensive molecules, is that they target the fundamental design differences between microbial membranes and the membranes of plants and animals, therefore the emergence of resistant strains of the pathogens is less probable compared to that of conventional antibiotics. In addition, AMPs can be easily modified *in vitro* based on principles of peptide modeling to improve their activities (*5*). Because of their broad-spectrum antimicrobial activity and low cytotoxicity towards mammalian cells, these "little wonders" are considered as a new generation of potent pharmaceuticals that can address the ever-growing problem of antibiotic resistance, eventually replacing the traditional antibiotics.

The vast majority of research on AMPs has focused on their activities against pathogens of medical importance, with the ultimate goal of developing human therapeutics. The data obtained in medical studies have generated a great interest in expanding the application of AMPs to a new area, namely plant biotechnology, as an innovative strategy for engineering broad-spectrum disease resistance in plants, as well as an opportunity to reduce the production cost of these peptides for human and animal needs (molecular farming).

The intention of this chapter is to review recent data from our lab on expression of natural and synthetic AMPs in a variety of plant species, and regulated by different promoters. We discuss their activities against plant-specific pathogens *in vitro*, as well as their cytotoxicity towards host plants. The levels of transgene expression and peptide accumulation that are sufficient to confer resistance against broad range of phytopathogens are examined. Additionally, current challenges in applying AMPs for crop improvement are described, along with a discussion of future prospects.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

Activity of Antimicrobial Peptides against Phytopathogens *in Vitro*

Our lab has a long and successful history of expressing AMPs in plants (Table 1). The peptides were selected from a pool of natural AMPs, or developed *de novo* based on careful analyses of their structure and activities *in vitro*. Many AMPs derived from our long-term collaboration with Dr. Robert Hancock from the University of British Columbia, Canada. Before the AMP genes were transferred into plants, the peptides were evaluated *in vitro* to obtain preliminary evidence for their use in protecting plants against economically important pathogens, such as *Fusarium*, *Alternaria*, *Botrytis*, *Cercospora*, *Phytophthora*, *Rhizoctonia*, *Septoria*, *Pectobacterium*, and others. Dose-response curves were obtained for each peptide-pathogen combination after 24 h incubation of fungal conidia or bacterial cells with different AMP concentrations. All tested pathogens were found to be sensitive to the AMPs at low micromolar peptide concentrations, with minimal inhibitory concentrations (MIC; defined as the lowest peptide concentration that completely inhibits the germination of conidia or bacterial growth) ranging from 2.5 to 10 μM for most peptide-pathogen combinations. Similar to other studies of AMPs (*6*, *7*), notable anomalies in hyphal morphology were observed at concentrations that only partially inhibited the germination of conidia (below the MIC). It included hyperbranching of the hyphae, swelling, and formation of condensed cells, in contrast to untreated controls. Thus, even sublethal concentrations of AMPs inhibited pathogen growth.

When AMPs were incubated with pathogenic fungi for longer periods (more than two days), some peptides (i.e., cecropin A-melittin derivative MsrA1 and CEMA) partially lost their potencies at concentrations below MIC, presumably due to the degradation by secreted pathogen proteinases. On the other hand, MsrA2 and temporin A were relatively resistant to pathogen proteinases, and even boiling did not inactivate them (*8*).

Phytotoxicity of Antimicrobial Peptides

To benefit their expression in plants, AMPs should have no adverse effect on the host organism. The phytotoxicities of candidate peptides were tested and compared *in vitro* using protoplasts, seedling roots, and intact leaf tissues of their respective hosts. Liquid cultures of freshly isolated mesophyll protoplasts were found to be the most sensitive indicators of AMP phytotoxicity. Another sensitive bioassay uses pollen (*9*), though such material may not be readily available throughout the year. In our work, protoplast viability was determined after incubation of cells with different amounts of AMPs for 24 h, followed by staining of the cell culture with neutral red or Evan's blue dye: the former accumulated in the vacuoles of living cells, whereas the latter stained dead cells. The threshold for peptide concentration that is toxic to plant cells varied among the AMPs and overall correlated with their antimicrobial activities against plant pathogens: more potent AMPs were also more toxic to plant protoplasts. Nevertheless, the minimal detectable phytotoxic concentration of each AMP was at least several-fold higher than the peptide levels required to completely inhibit the germination of conidia and bacterial growth. Interestingly, bioassays of three different human cell lines, cultured in the presence of temporin A, BMAP-18, and indolicidin variants 10R and 11R, showed that the cytotoxicities of these peptides toward mammalian cells were comparable to plant protoplasts. Seedling roots and leaf explants were even more tolerant to AMPs than protoplasts, and no visible toxic effects on plant tissue viability were observed up to the highest AMP concentrations tested (100 μM MsrA1 and 120 μM temporin A).

Table 1. Natural and synthetic antimicrobial peptides used in our lab for expression in plants

Peptide name	*Description*	*Amino acid sequence*[a]	*Reference*
CEMA	cecropin A-melittin hybrid	MKWKLFKKIGI-GAVLKVLTTGLPALKLTK	(*19*)
MsrA1	extended cecropin A-melittin hybrid	MALEHMKWKLFKKIGI-GAVLKVLTTGLPALKLTK	(*14*)
MsrA2	derivative of dermaseptin B1	MAMWKDVLKKIGTVAL-HAGKAALGAVADTISQ	(*8*, *22*, *31*)
MsrA3	extended temporin A	MASRHMFLPLIGRVLSGIL	(*15*)
Temporin A		MFLPLIGRVLSGIL	(*8*)
10R	indolicidin variant	MRRPWKWPWWPWRR	(*33*)
11R	indolicidin variant	MRWRRWPWWPWRRK	(*33*)
PV5	polyphemusin I variant	MRRWCFRVCYRGRFCYRKCR	(*32*)
PV8	polyphemusin I variant	MFRWCFRVCYKGRCRYKCR	(*49*)
BMAP-18	bovine myeloid AMP	MGRFKRFRKKFKKLFKKLS	(*34*)

[a] For expression in plants, amino acid sequences of all peptides were preceded by methionine.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

Inducible versus Constitutive Expression of Antimicrobial Peptides

High antimicrobial activities *in vitro*, combined with low phytotoxicities, have prompted experiments on the expression of these AMPs in plants. Plant-optimized AMP-coding nucleotide sequences were transcriptionally fused to a designated promoter, and introduced into plants via *Agrobacterium*-mediated transformation. The transformed plant species included potato (*Solanum tuberosum* L, cvs Russet Burbank and Desiree), tobacco (*Nicotiana tabacum* L. cv. Xanthi), hybrid poplar (*Populus nigra* L. x *Populus maximowiczii* A. Henry, genotype NM6), rapeseed (*Brassica napus* L.), and arabidopsis (*Arabidopsis thaliana* L.). In addition to

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

the AMP properties, the choice of a suitable promoter with the desired level of spatial and temporal activity is an important factor for the successful engineering of AMP-expressing plants. In most studies, the strong constitutive *CaMV 35S* promoter and its derivatives were used in regulating the expression of AMP genes. However, permanent long-term exposure of a host plant to high levels of AMPs, caused by nonstop activity of the constitutive promoter, may negatively affect plant functions even at peptide concentrations that were initially shown to be non-phytotoxic. Also, the constitutive expression of defensive compounds creates an undesirable selection pressure on pathogen populations, which may eventually lead to the development of resistant strains (*10*). Most importantly, constitutive promoters of non-plant origin are frequently linked to transgene silencing (*11*, *12*), which reduces the amount of AMPs in plants to a level insufficient to kill the pathogen. The low level of AMPs in transgenic plants, and subsequently, the low disease resistance observed in some studies on the expression of natural and synthetic cecropins in plants, has been suggested to be the result of not only the proteolytic activity of the host proteases, but also the use of the constitutive viral promoter (*13*). Consequently, the use of an inducible plant promoter that activates only in response to pathogen invasion or pest attack, especially in a predictable spatio-temporal manner, has clear advantages for engineering disease resistance in plants. On the other hand, the use of constitutive promoters is more preferable for molecular farming; i.e., when the goal is to reach the maximum accumulation of AMPs in plant tissues.

Although we have used constitutive promoters to improve host resistance against microbial diseases (*14*, *15*), our recent studies on pathogen-resistant plants have focused on the expression of AMP genes under control of inducible promoters, particularly, the *win3.12 T* promoter from hybrid poplar (*Populus trichocarpa* X *P. deltoides*). This promoter is a truncated version of the upstream region of the wound-inducible *win3.12* gene (*16*, *17*), which encodes a Kunitz-type proteinase inhibitor. We have shown that the *win 3.12T* poplar promoter contains several pathogen-responsive *cis*-acting elements, exhibits strong systemic activity in plants in response to mechanical wounding and infections with a variety of pathogens, and is thought to be a part of the poplar defense system (*18*, *19*). The nucleotide sequences of cecropin A-melittin, MsrA2, and temporin A were optimized for expression in plants, and transcriptionally fused to the *win3.12T* promoter. The expression vectors were introduced into genomes of potato (Figure 1A), tobacco (*8*, *19*), and hybrid poplar (Figure 1B). Northern analysis and quantitative RT-PCR of DNase-treated leaf RNA from transgenic plants confirmed the rapid accumulation of AMP transcripts in response to pathogen infection, whereas in leaves of unstressed plants it was almost undetectable. Upon induction, the amount of AMPs was up to 6-7 μg per gram of fresh leaf tissue. The *win3.12T*-driven accumulation of AMPs in transgenic plants had no deleterious effect on plant growth and development. Most importantly, the expression level of the AMPs *in vivo*, regulated by the *win3.12T* promoter, was sufficient to confer plant resistance against a wide range of pathogenic fungi, oomycetes, and bacteria. The AMPs used in this research differed from each other in antimicrobial potencies and sensitivity to proteinase degradation. Nevertheless, direct comparisons of disease resistance among the best transgenic

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

lines expressing one of the following AMPs: cecropin A-melittin, MsrA2, or temporin A peptides, have shown that despite some variability, a sufficient level of AMP, rather than the type of AMP, was the deciding factor for the ability of transgenic plants to fight pathogens and withstand infections (Figure 2).

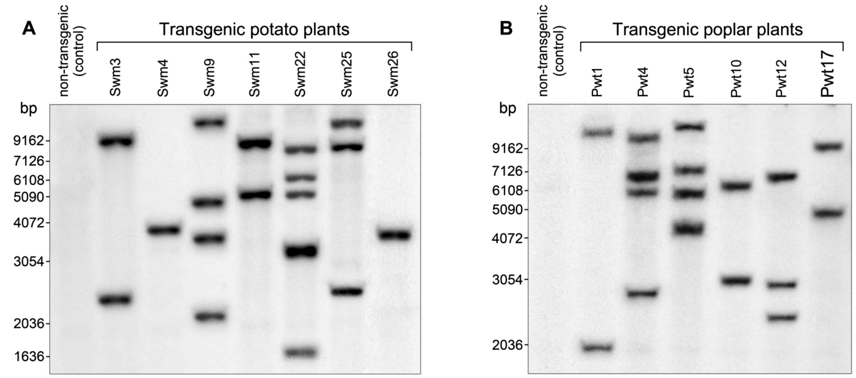


Figure 1. Southern blot analyses of potato plants transformed with MsrA2 gene (A), and poplar plants transformed with temporin A gene (B). In both experiments, the expression of heterologous AMPs was regulated by the pathogen-inducible win3.12T promoter from hybrid poplar. Plant DNA was digested with XbaI, electrophoresed, and hybridized with ^{32}P-labelled antisense DNA strand of either the MsrA2 (A) or temporin A (B) gene. The antisense DNA probes were prepared by linear PCR amplification using transgene-specific reverse primers. The number of bands in each lane reflects the number of transgene insertions in the corresponding transgenic line (plant). The transgene copy number in bands with higher signal intensity was determined by a Molecular Dynamics densitometer. Molecular weight DNA markers are shown on the left of each autoradiogram.

Organ-Specific Expression of Antimicrobial Peptides

The development of crops with reduced post-harvest losses requires a targeted organ-specific accumulation of AMPs, accompanied with the precise control of spatial transgene expression. An efficient combination of promoter and AMP-coding gene with a suitable level of organ-specific expression is a prerequisite for practical applications of this technology. In our studies, comparative analyses of different promoters in plants using β-glucuronidase (GUS) reporter gene system revealed that the promoter of luminal binding protein from Douglas-fir (*BiP Pro1-1* promoter) exhibited high activity in potato tubers. This encouraged us to evaluate the use of this promoter for accumulation of an antimicrobial peptide MsrA2 in storage organs of potato. The MsrA2 peptide (32 amino acids) is a derivative of dermaseptin B1 that was originally isolated from the skin secretion of the arboreal frogs *Phyllomedusa sauvagei* and *P. bicolor* (*20*, *21*). It has powerful antimicrobial activity, inhibiting numerous species of plant pathogenic fungi and bacteria not only *in vitro*, but also *in vivo* (*8*). Combined with low toxicity to mammalian and plant cells, MsrA2 presents a great potential for engineering plants with increased disease resistance. The nucleotide sequence encoding MsrA2 was transcriptionally fused to the *BiP Pro1-1* promoter, and introduced into potato via *Agrobacterium*-mediated transformation (*22*). Stable transgene integration into the plant genome was confirmed with Southern analysis. Growth characteristics and the morphology of transgenic potato plants were identical to the untransformed controls. Western blot analysis showed a high level of MsrA2 accumulation in the tubers of transgenic plants, up to 8 µg per gram of raw tuber tissue. The expression level of the MsrA2 peptide in potato tubers, regulated by the *BiP Pro1-1* promoter, was sufficient to confer resistance against bacterial soft rot disease caused by plant-pathogenic species of *Erwinia* (*Pectobacterium carotovorum*). Moreover, the tubers retained their resistance to the bacterial disease for more than a year. Thus, the tuber-specific expression of MsrA2 enables the engineering of soft rot-resistant potato plants, thereby reducing tuber losses in the post-harvest system. The expression of AMPs in organs such as potato tubers may also be used for developing plant-derived edible vaccines (or biopharmaceuticals in general).

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

Increased Productivity of Transgenic Plants Expressing Antimicrobial Peptides

While testing the disease resistance of potato plants grown in greenhouse, we discovered an interesting phenomenon: the tuber yield of AMP-expressing plants was always 15-25% higher than that of untransformed controls. The results described both the weight and size of tubers produced by either individual transgenic plants or group of plants of the same line. Moreover, the higher tuber yield was observed in all transgenic plants, irrespective of the AMP type expressed (MsrA1, MsrA2, or MsrA3). These results were consistent during six consecutive growing seasons of the plants. The enhanced yield of transgenic tubers in the absence of disease was confirmed in a more detailed study of potato that contained the MsrA3 peptide ((*23*); Goyal, R. K. University of Victoria, BC, Canada; Hancock, R. E. W. University of British Columbia, Vancouver, Canada; Misra, S. University of Victoria, BC, Canada; *unpublished*). It is hypothesized that in addition to immediate antimicrobial activity, the expression of at least some AMPs in plants modulates components of the natural host defense in a way that significantly increases the productivity of transgenic plants, even in the absence of biotic stress. Indeed, the expression of AMP in plants resulted in a suppressed activation of hypersensitive response (HR) during both biotic and abiotic stresses. Because HR is resource-intensive, its activation has a negative effect on photosynthesis and overall plant productivity (*24*). Therefore, the suppression of HR in transgenic AMP plants likely diverts some resources from HR-mediated defense management to plant productivity, which in turn resulted in the significantly higher yield of tubers. Furthermore, these plants displayed increased longevity under stress. Thus, the expression of heterologous AMPs in plants not only provides powerful broad-spectrum disease-resistance, but also enhances innate immunity resulting in increased yield and productivity.

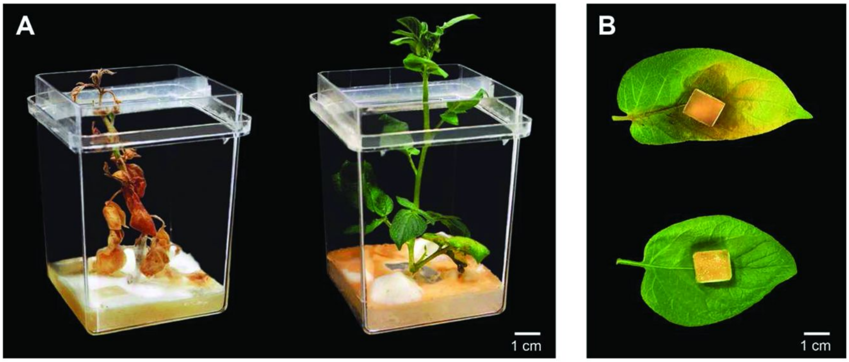


Figure 2. (A) Resistance of potato plants to the pathogenic fungus Fusarium solani. Two 1-cm^2 agar blocks of the fungal mycelia were placed 2 cm from the stem of a well-developed potato plant grown in vitro, and cultivated for several weeks. The bioassay was based on the ability of resistant plants to survive infections much longer than untransformed plants. Control and MsrA2-expressing plant Swm22 (on the right) are shown after 12 days of co-cultivation with the pathogen. The expression of MsrA2 was regulated by the pathogen-inducible win3.12T promoter from hybrid poplar. (B) Resistance of detached potato leaves to the plant pathogenic fungus Verticillium sp. Fully expanded young potato leaves were excised from 2-month-old plants grown in the greenhouse, and placed on sucrose-free MS agar medium with the adaxial side up. A 1-cm^2 agar block of freshly grown Verticillium culture was placed in the center of the detached leaf with the mycelium side down, in direct contact with the plant tissue. The leaves were cultivated under standard plant tissue culture conditions, and observed daily for disease symptoms. Control and temporin A-expressing leaf from plant Sbtt17 (on the bottom) are shown 8 days after inoculation with Verticillium sp.. The expression of temporin A was regulated by the truncated promoter of luminal binding protein from Douglas-fir (BiP Pro1-3 promoter). Both Fusarium solani and Verticillium sp. were obtained from fungal culture collection of Dr. Zamir Punja (Simon Fraser University, Burnaby, BC, Canada). (see color insert)

Production of Antimicrobial Peptides in Transgenic Plants for Pharmaceutical Use

To fully exploit the unique qualities of AMPs to treat bacterial infections in humans and animals, the production of AMPs must be economically feasible. Despite significant cost reductions, the traditional methods for making AMPs by *de novo* chemical peptide synthesis remain prohibitively expensive. A growing

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

interest has been shown in transgenic plant technology to meet industry demand for AMPs. Using plants as a platform for the efficient production of heterologous bio-products offers many benefits, with the main advantage being the production of any given recombinant peptide or protein at an agricultural scale and for a relatively low cost, without risks of contamination by endotoxins or human pathogens. Currently, the limitations of plant-based production systems include the low yields of many proteins (usually caused by poor protein stability), and difficulties with the downstream processing. The general approach to improve protein yields is to maximize the efficiency at all stages of transgene expression and to improve protein stability by appropriate subcellular targeting. The right choice of host species, a productive expression system (e.g., stable transformation vs. transient expression, nuclear vs. chloroplast transformation), as well as efficient promoter-transgene constructs with a high level of organ- or tissue-specific expression are the essentials to achieve a commercially feasible level of AMPs in plants. We have explored the possible use of transgenic poplar, which is a non-food plant with a fast growth rate, for high-level accumulation of the dermaseptin B1 derivative, MsrA2. Despite activity on bacterial membranes, dermaseptins do not lyse erythrocytes or other mammalian cells (*25*), and therefore present great potential use as pharmaceuticals. The MsrA2-coding gene was inserted into the constitutive, high expression promoter cassette, which incorporated a duplicated-enhancer *CaMV 35S* promoter with a *cis*-active translational activator sequence from alfalfa mosaic virus (*26*), and introduced into the genome of a commercial hybrid poplar *P. nigra* x *P. maximowiczii*, using *Agrobacterium*. Genomic DNA from the plants regenerated on selection medium was analyzed for the presence of the *MsrA2* gene (Figure 3A), its correct transcription and translation into active MsrA2 peptide (Figure 3B). Among all organs tested, the highest level of *MsrA2* mRNA was detected in leaves of transgenic poplars. To confirm that *MsrA2* transcripts were correctly processed into peptides and not degraded by host proteases, protein extracts from the leaves of transgenic plants were examined with Western blot analysis using polyclonal antibodies raised against MsrA2 peptide. Comparisons of the signal intensity of protein samples from transgenic plants with synthetic MsrA2 of known concentration indicated that the accumulation of the MsrA2 peptide in the leaves of transgenic poplars ranged from 2 to 6 μg per gram of fresh tissue (Figure 3B). *In vitro* bioassays confirmed that MsrA2 was expressed in a form that retained its native antimicrobial properties. The morphological characteristics of transgenic poplars were comparable to those of control plants, with no indication of cytotoxicity from the accumulation of the peptide. Since an average yield of fresh leaves from a 2-year-old plantation of *P. deltoides* (another commercial poplar) is 8,600 kg per hectare (*27*), the total amount of MsrA2 in all transgenic leaves can be easily estimated to determine the economic feasibility of its downstream purification. Experiments on the purification of plant-derived MsrA2 and the overall peptide recovery are currently in progress. Overall, these studies provide insight into developing innovative technologies for the large-scale production of AMPs and other heterologous compounds. Alternative methods for producing large quantities of AMPs in plants include transformation of chloroplasts (*28*) and transient expression using plant viral vectors (*29*, *30*).

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

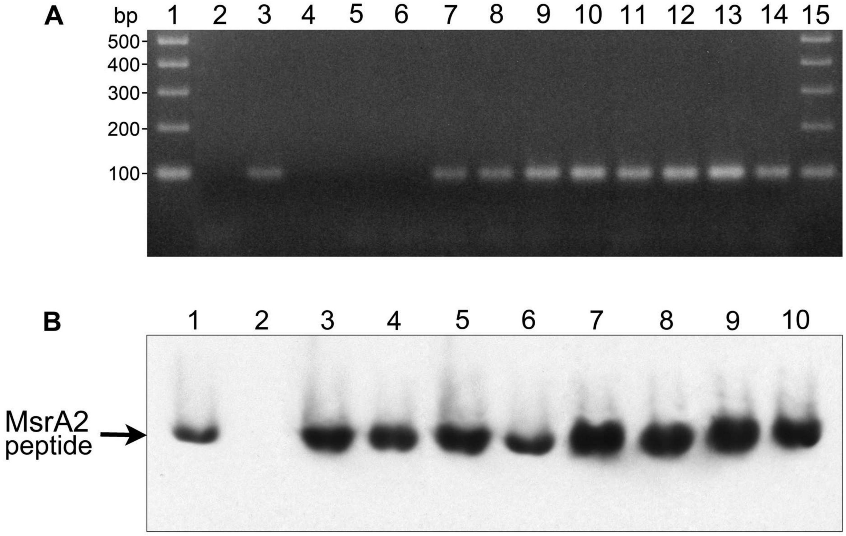


Figure 3. (A) Polymerase chain reaction (PCR) analysis of DNA isolated from poplar lines transformed with MsrA2 gene. Fragments of 99 bp were generated using MsrA2-specific primers, and indicated the presence of the transgene (the 96 bp coding region with the TAA stop codon). Lanes 1 and 15: 100-bp DNA ladder. Lane 2: PCR mix without template DNA. Lane 3: plasmid, containing MsrA2 gene (positive control). Lane 4: PCR mix without 5'MsrA2 forward primer. Lane 5: PCR mix without 3'MsrA2 reverse primer. Lane 6: untransformed poplar (control). Lanes 7-14: transgenic poplar lines Pm9, Pm11, Pm15, Pm16, Pm20, Pm22, Pm25, and Pm28, respectively. (B) Western blot analysis of the accumulation of MsrA2 peptide in transgenic poplars. Proteins were isolated from the leaves of poplar plants used in the PCR analysis above, separated by acid urea polyacrylamide gel electrophoresis, transferred to a Hybond-P PVDF membrane, and hybridized with MsrA2-specific polyclonal antibodies. Lane 1: synthetic MsrA2 peptide (200 ng, positive control). Lane 2: untransformed poplar (control). Lanes 3-10: transgenic poplar lines Pm9, Pm11, Pm15, Pm16, Pm20, Pm22, Pm25, and Pm28, respectively. The expression of MsrA2 was regulated by the constitutive, duplicated-enhancer CaMV 35S promoter with AMV translational activator.

Conclusions and Future Prospects

The remarkable properties of antimicrobial peptides and their primary roles in host defense systems make them excellent candidates for heterologous expression in plants, especially when the objective is to create plants with broad-spectrum disease resistance. Traditionally, the protection of plants against pathogenic microorganisms has been pursued through development of resistant cultivars using conventional breeding, or by the extensive use of pesticides. Both of these

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

approaches have significant limitations: the former due to interspecific sexual incompatibility and the lack of a desired gene pool in donor species, as well as the need for numerous time-consuming back-crossings, whereas the latter has a highly negative long-term impact on the environment. Current methods of genetic engineering have dramatically changed breeding technologies, providing a unique opportunity to introduce virtually any gene of interest into plant genome without altering valuable traits of the otherwise perfect cultivars. Transformation of plants with genes encoding the AMPs is a promising strategy to effectively fight plant diseases. Most of the AMPs tested in our lab combined powerful antimicrobial activities with low phytotoxicities at physiologically important concentrations. Their expression in plants, especially when regulated by a strong pathogen-inducible promoter, provides a safe and efficient mechanism to enhance plant resistance against a wide range of pathogenic fungi, oomycetes, and bacteria (*8*, *14*, *15*, *19*, *31–34*). To date, a number of AMP-expressing plant species have been developed that confer different degrees of protection against important phytopathogens (*27*, *35–43*). The challenges of expressing AMPs for plant protection include instability of some peptides due to relatively quick degradation by host proteases, which decreases their efficiency during peptide-pathogen interactions (*44–46*), and their insufficient level of expression caused by transgene silencing or poor promoter choice. Rational design and molecular modeling would seem to be efficient methods for improving AMP stability without compromising antimicrobial activity (*5*). The biggest challenge for the expression of AMPs in plants, however, is the public opposition to transgenic technologies in general, though no valid evidence has been found to suggest that these plants may be harmful to human health. To the contrary, AMPs are natural compounds that are produced in all organisms to fight pathogen invasion, and their expression in commercial crops can reduce the use of pesticides in the environment and the associated health risks to humans. The increased productivity of transgenic AMP plants suggests that these peptides act as multifunctional molecules and may have other, unknown functions in plants associated not only with defense, but also with plant development and abiotic stresses. This is a major breakthrough in current AMP research for plant improvements that has a potential to reduce field and storage losses, provide safer foods, and improve crop yields.

Future areas of interest include the simultaneous expression in plants of more than one type of AMP, preferably with different modes of action, to further improve broad-spectrum activity against diverse groups of plant pathogens, and to minimize the probability of a pathogen overcoming engineered resistance. The synergistic action of AMPs has been shown *in vitro* (*47*), but remains to be confirmed *in planta*. Other prospects include approaches that rely on peptide chemistry to develop new, synthetic AMPs that are superior to their counterparts from natural sources; for example, with improved pathogen-specific activity, decreased cytotoxicity, and appropriate levels of protease-related stability. As for the control of heterologous AMP genes in plants, the technology is clearly evolving towards using pathogen-inducible or organ-, tissue-specific promoters that drive AMP expression only when and where needed. Also, the engineered plant resistance to pathogens has been mostly tested under controlled laboratory conditions *in vitro*, or in greenhouse. It is therefore important to extend the evaluation of transgenic AMP plants to field trials to ensure that this technology can be successfully implemented in disease management programs. Another increasingly popular area to explore is the use of transgenic plants as factories to reduce production costs for AMPs targeted for pharmaceutical use. The magnifection process, which is based on transient expression technology using plant viral vectors (*48*), appears to be the most promising plant-based production platform to obtain the highest yields of recombinant AMPs.

In summary, our studies have demonstrated enormous potentials for AMPs in controlling plant diseases, providing crops with higher yields, improving food quality, and reducing the cost of pharmaceuticals. In the next decade, we will likely see an abundance of new studies that will broaden our knowledge and stimulate the practical use of these small, yet powerful molecules.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

References

1. Zasloff, M. *Nature* **2002**, *415*, 389–395.
2. Hancock, R. E. W.; Scott, M. G. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8856–8861.
3. Hancock, R. E. W.; Diamond, G. *Trends Microbiol.* **2000**, *8*, 402–410.
4. Papo, N.; Shai, Y. *Biochemistry* **2004**, *43*, 6393–6403.
5. Marcos, J. F.; Muñoz, A.; Pérez-Payá, E.; Misra, S.; López-García, B. *Annu. Rev. Phytopathol.* **2008**, *46*, 273–301.
6. Cavallarin, L.; Andreu, D.; San Segundo, S. *Mol. Plant-Microbe Interact.* **1998**, *11*, 218–227.
7. Ali, G. S.; Reddy, A. S. N. *Mol. Plant-Microbe Interact.* **2000**, *13*, 847–859.
8. Yevtushenko, D. P.; Misra, S. *Plant Biotechnol. J.* **2007**, *5*, 720–734.
9. Jacobi, V.; Plourde, A.; Charest, P. J.; Hamelin, R. C. *Can. J. Bot.* **2000**, *78*, 455–461.
10. Jouanin, L.; Bonadé-Bottino, M.; Girard, C.; Morrot, G.; Giband, M. *Plant Sci.* **1998**, *131*, 1–11.
11. Matzke, M. A.; Matzke, A. J. M. *Plant Physiol.* **1995**, *107*, 679–685.
12. Puddephat, I. J.; Riggs, T. J.; Fenning, T. M. *Mol. Breeding* **1996**, *2*, 185–210.
13. Yi, J. Y.; Seo, H. W.; Yang, M. S.; Robb, E. J.; Nazar, R. N.; Lee, S. W. *Planta* **2004**, *220*, 165–171.
14. Osusky, M.; Zhou, G.; Osuska, L.; Hancock, R. E. W.; Kay, W. W.; Misra, S. *Nat. Biotechnol.* **2000**, *18*, 1162–1166.
15. Osusky, M.; Osuska, L.; Hancock, R. E. W.; Kay, W. W.; Misra, S. *Transgenic Res.* **2004**, *13*, 181–190.
16. Hollick, J. B.; Gordon, M. P. *Plant Mol. Biol.* **1993**, *22*, 561–572.
17. Hollick, J. B.; Gordon, M. P. *Plant Physiol.* **1995**, *109*, 73–85.
18. Yevtushenko, D. P.; Sidorov, V. A.; Romero, R.; Kay, W. W.; Misra, S. *Plant Sci.* **2004**, *167*, 715–724.
19. Yevtushenko, D. P.; Romero, R.; Forward, B. S.; Hancock, R. E. W.; Kay, W. W.; Misra, S. *J. Exp. Bot.* **2005**, *56*, 1685–1695.
20. Mor, A.; Amiche, M.; Nicolas, P. *Biochemistry* **1994**, *33*, 6642–6650.

21. Fleury, Y.; Vouille, V.; Beven, L.; Amiche, M.; Wróblewski, H.; Delfour, A.; Nicolas, P. *Biochim. Biophys. Acta* **1998**, *1396*, 228–236.
22. Yevtushenko, D. P.; Misra, S. In *The Canadian Society of Plant Physiologists Annual Meeting*; Halifax, Canada, 2011.
23. Goyal, R. K.; Hancock, R. E. W.; Misra, S. In *The Advanced Foods and Materials Network Annual Scientific Conference*; Guelph, Canada, 2009.
24. Swarbrick, P. J.; Schulze-Lefert, P.; Scholes, J. D. *Plant Cell Environ.* **2006**, *29*, 1061–1076.
25. Strahilevitz, J.; Mor, A.; Nicolas, P.; Shai, Y. *Biochemistry* **1994**, *33*, 10951–10960.
26. Datla, R. S. S.; Bekkaoui, F.; Hammerlindl, J. K.; Pilate, J.; Dunstan, D. I.; Crosby, W. L. *Plant Sci.* **1993**, *94*, 139–149.
27. Szekai, Z,; Fengjue, L.; Daoqun, Z.; Qingxiao, L.; Xiazi, W.; Xinsheng, M.; Zhenlei, Z. In *Agroforestry Systems in China;* Zhaohua, Z., Mantang, C., Shiji, W., Youxu, J., Eds; The Chinese Academy of Forestry and IDRC: Singapore, 1998; pp 113−115.
28. DeGray, G.; Rajasekaran, K.; Smith, F.; Sanford, J.; Daniell, H. *Plant Physiol.* **2001**, *127*, 852–862.
29. Gelvin, S. B. *Nat. Biotechnol.* **2005**, *23*, 684–685.
30. Gleba, Y.; Klimyuk, V.; Marillonnet, S. *Curr. Opin. Biotechnol.* **2007**, *18*, 134–141.
31. Osusky, M.; Osuska, L.; Kay, W. W.; Misra, S. *Theor. Appl. Genet.* **2005**, *111*, 711–722.
32. Bhargava, A.; Osusky, M.; Forward, B. S.; Hancock, R. E. W.; Kay, W. W.; Misra, S. *Plant Cell Tissue Organ Cult.* **2007**, *88*, 301–312.
33. Bhargava, A.; Osusky, O.; Hancock, R. E. W.; Forward, B. S.; Kay, W. W.; Misra, S. *Plant Sci.* **2007**, *172*, 515–523.
34. Francescutti, T. M. M.S. thesis, University of Victoria, Victoria, Canada, 2007.
35. Jaynes, J. M.; Nagpala, P.; Destefano-Beltran, L.; Huang, J. H.; Kim, J. H.; Denney, T.; Cetiner, S. *Plant Sci.* **1993**, *89*, 43–53.
36. Huang, Y.; Nordeen, R. O.; Di, M.; Owens, L. D.; McBeath, J. H. *Phytopathology* **1997**, *87*, 494–499.
37. Mitsuhara, I.; Matsufuru, H.; Ohshima, M.; Kaku, H.; Nakajima, Y.; Murai, N.; Natori, S.; Ohashi, Y. *Mol. Plant-Microbe Interact.* **2000**, *13*, 860–868.
38. Gao, A.; Hakimi, S. M.; Mittanck, C. A.; Wu, Y.; Woerner, B. M.; Stark, D. M.; Shah, D. M.; Liang, J.; Rommens, C. M. T. *Nat. Biotechnol.* **2000**, *18*, 1307–1310.
39. Sharma, A.; Sharma, R.; Imamura, M.; Yamakawa, M.; Machii, H. *FEBS Lett.* **2000**, *484*, 7–11.
40. Chakrabarti, A.; Ganapathi, T. R.; Mukherjee, P. K.; Bapat, V. A. *Planta* **2003**, *216*, 587–596.
41. Mentag, R.; Luckevich, M.; Morency, M.-J.; Séguin, A. *Tree Physiol.* **2003**, *23*, 405–411.
42. Rajasekaran, K.; Cary, J. W.; Jaynes, J. M.; Cleveland, T. E. *Plant Biotechnol. J.* **2005**, *3*, 545–554.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

43. Coca, M.; Peñas, G.; Gómez, J.; Campo, S.; Bortolotti, C.; Messeguer, J.; Segundo, B. S. *Planta* **2006**, *223*, 392–406.
44. Hightower, R.; Baden, C.; Penzes, E.; Dunsmuir, P. *Plant Cell Rep.* **1994**, *13*, 295–299.
45. Allefs, S. J. H. M.; Florack, D. E. A.; Hoogendoorn, C.; Stiekema, W. J. *Am. Potato J.* **1995**, *72*, 437–445.
46. Florack, D.; Allefs, S.; Bollen, R.; Bosch, D.; Visser, B.; Stiekema, W. *Transgenic Res.* **1995**, *4*, 132–141.
47. Westerhoff, H. V.; Zasloff, M.; Rosner, J. L.; Hendler, R. W.; De Waal, A.; Vaz Gomez, A.; Jongsma, P. M.; Riethorst, A.; Juretic, D. *Eur. J. Biochem.* **1995**, *228*, 257–264.
48. Gleba, Y.; Klimyuk, V.; Marillonnet, S. *Vaccine* **2005**, *23*, 2042–2048.
49. Misra, S.; Bhargava, A. In *Biotechnology and Plant Disease Management*; Punja, Z. K., De Boer, S. H., Sanfacon, H., Eds; CABI: Wallingford, U.K., 2007; pp 301−320.

Downloaded by CORNELL UNIV on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ch021

Subject Index

A

- AGM 184 peptide, 301*f*
- Alpha helical conformation-specific antibodies development
  - GCN4 stabilized HRC, 117
  - HRC, 102, 116, 120
  - HRC antigenicity, 122
  - HRC immunogenicity, 109, 122
  - HRC recognition, 124
  - peptide-based vaccine, 103
  - SARS-CoV infectivity neutralization, 111
  - SARS-CoV S protein, 101
  - synthesis, 103
  - synthetic HRC, 108
  - two-stranded coiled-coil template, 125
  - two-stranded peptide immunogens, 110
  - virus entry, 101
- α-defensins, 153
- α-helical coiled-coil template, 106*f*, 107*f*
- αHTH
  - Arabidopsis transgenic lines, 438*t*
  - bilayer leaflet, interactions, 428*f*
- αHthΔ, thionin antifungal peptide synthesis, 373
- αHth1, thionin antifungal peptide synthesis, 373
- αHthΔ mRNA translation analysis, thionin antifungal peptide synthesis, 365*f*, 366, 368*f*
- Altered Hth1, thionin antifungal peptide synthesis, 365
- Alyteserin-1c, 54
- Amino acid sequence
  - antifungal peptides, 339*f*
  - lytic peptides, 34*t*
- Amino-terminus, lytic peptides, 26*f*
- AMP. *See* antimicrobial peptides (AMP)
- Angiogenesis, lytic peptides, 28, 30*f*
- Animal gut microbiota control, AMP, 218
  - drosophila gut, 218
  - mammalian gut, 220
- Antibacterial resistance, 437*f*
- Antibiotics, 420*t*
- Antibody labelling, 196
- Anticancer activity, lytic peptides, 27
- Anticancer therapeutics, lytic peptides
  - D2A21, 85*f*
  - design principles, 80
  - interpolated mean IC50 values, 87*t*
  - lysis sensitivity, 82*f*
  - overview, 79
  - set sequence, 83*t*
  - systemic anticancer activity, 86
  - tumor targeting, 87
  - in vitro activity, 81
  - in vivo activity, 86
- Antifungal activity, antifungal plant defensins, 326*f*, 328*f*
- Antifungal peptides
  - amino acid sequence, 339*f*
  - cell penetration properties, 345
  - fluorescently labeled, 346*f*
  - fungi interation, 342
  - intracellular killing, 348
  - non-lytic modes, 342
  - overview, 337
- Antifungal plant defensins
  - antifungal activity, 326*f*, 328*f*
  - antifungal properties, 323
  - conidial germination inhibition, 323*f*
  - expression, 318, 330
  - fungal resistance, 330
  - modes of action, 324
  - oomycete pathogen resistance, 330
  - overview, 317
  - pathogens, 320*t*
  - structure, 318
  - structure-activity relationships, 324
  - transgenic crops, 330
- Antifungal properties, antifungal plant defensins, 323
- Anti-HRC antibodies, 114*f*
- Antimicrobial peptides (AMP), 2, 5, 420*t*, 421*f*
  - animal gut microbiota control, 218
    - drosophila gut, 219
    - mammalian gut, 220
  - fire blight control
    - antibacterial activity, 403*t*
    - cecropin-melitting hybrids against *E. amylovora*, 410
    - *E. amylovora*, 406, 408*f*
    - iminopyrrolidine-containing peptides, 404, 405*f*
    - magainins hybrids against *E. amylovora*, 410
    - New Zealand, 401
    - overview, 397
    - Pantocin, 408, 409*f*
    - piperidine-containing peptides, 404, 405*f*
    - pyrazole-conjugated peptides, 402

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ix002

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ix002

    streptomycin, 407*f*
  microbial symbiont control, 216
  natural and synthetic, 448*t*
  nitrogen fixing endosymbionts
    bacterial differentiation, 227
    bacterial protection, 225
    NCR peptide family, 223
    NCR peptide interaction, 224
    symbiotic bacterial population establishment, 222
    symbiotic rhizobium bacteria differentiation, 222
  overview, 215
  pathogen fighting, 218
  plant disease control
    activity screening, 240
    additional key properties screening, 243
    CECMEL11, 244, 247
    CYC10, 244, 247
    cyclic decapeptides, 244*f*
    flow diagram, 239*f*
    linear undecapeptides, 244*f*, 250*t*, 251*t*
    living organisms, 237
    overview, 235
    pathogen killing, 255*f*
    peptide properties optimization, 247*f*
    production costs, 254
    proof-of-concept, 252
    synthesis optimization, 254
    synthetic derivatives, 239
    toxicology, 253
  plant disease protection
    β-barrelins, 272
    biological function, 273
    biotechnological applications, 274
    cyclotides, 270
    four-cysteine, 272
    glycine-rich cysteine-free, 273
    hevein-like peptides, 269
    knottin-like family, 270
    LTP, 268
    overview, 263
    plant antimicrobial peptides, 266
    plant defensins, 267
    2S albumins, 272
    snakins, 269
    thionins, 266
    transgenic expression, 278
  symbiotic associations, 215
  transgenic expression, plants
    increased productivity, 451
    inducible versus constitutive, 448
    organ-specific, 450
    overview, 445
    pharmaceutical use, 452
    phytopathogens, 447
    phytotoxicity, 447
Antimicrobial synthetic peptides
  AGM 184 peptide, 301*f*
  bacterial pathogens, 302*t*
  D2A21 peptide, 299*f*
  D4E1 peptide, 299*f*, 301*f*, 305*f*
  fungal pathogens, 302*t*
  genesis, 298
    amphipathy, 300
    antimicrobial activity, 300
    charge density, 300
    hydrophobicity, 300
    mode of action, 301
  non-nuclear expression, transgenic plants, 306
    mitochondrial transformation, 307
    organelles, 308
    plasticid transformation, 307
    transgene expression, 307
    transgenic expression, 309
  nuclear expression, transgenic plants, 304
  overview, 295
Arabidopsis transgenic lines, 438*t*
Ascaphin-8, 54

B

Bacterial differentiation, 227
Bacterial pathogens, 302*t*
Bacterial protection, 225
β-barrelins, 272
Biological function, plant AMP, 273
Biotechnological applications, plant AMP, 274
Biotechnological approaches, plant AMP, 284
Brevinin-1BYa, 56
Brevinin-1PRa, 56
Brevinin-2-related peptide, 52
Broad spectrum antimicrobials, cationic peptides, 6

C

CAP. *See* cationic antimicrobial peptides (CAP)
Cationic antimicrobial peptides (CAP)
  anticancer properties, 63
  clinical use, 63
  modification, 63

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ix002

- hydrophobicity, 64
- motif addition, 66
- positive charge, 64
- overview, 61
- stability enhancement, 64*t*, 67
  - additional strategies, 72
  - cyclization, 69
  - D-amino acid incorporation, 71
  - end capping, 67
  - truncation, 70

CDNA library construction, thionin antifungal peptide synthesis, 372
CECMEL11, 244, 247
Cecropin B. Cells, 421*f*
Cecropin-melitting hybrids against *E. amylovora*, 410
Cell penetration properties, antifungal peptides, 345
Cereal thionins, thionin antifungal peptide synthesis, 360
Chemokines, 34
Circular dichroism spectroscopy, peptide immunogens, 129
Conidial germination inhibition, antifungal plant defensins, 323*f*
CYC10, 244, 247
Cyclic decapeptides, 244*f*
Cysteine residues, defensins, 138*f*
Cytokines
- overview, 21
- structural analysis, 34
- versus lytic peptides, 21, 24

D

D2A21 peptide, 299*f*
D4E1 peptide, 299*f*, 301*f*, 305*f*
Deduced amino acid sequences, thionin antifungal peptide synthesis, 363*f*
Defensins, 267
- biological functions, 140
- cell sources, 139
- clinical aspects
  - HIV pathogenesis, 153
- cysteine residues, 138*f*
- effect, viral infection
  - enveloped viruses, 144*t*
  - HIV, 143
  - HSV, 150
  - influenza virus, 150
  - non-enveloped viruses, 147*t*, 152
  - parainfluenza virus, 151
  - SARS-CoV, 151
  - vaccinia virus, 151
- human defensins, 138
  - α-defensins, 153
  - HBD, 155
  - levels, 153
- immunological functions, 140
- overview, 137
- regulation, 141
- structure-function relationship, 139
- tissue distribution, 139
- viral infection effect, 142

Designed AMP expression, *Theobroma cacao*
- antifungal activity, 383
- D4E1, 386*f*
- developmental stages, 384*f*
- disease suppression, 391
- gene encoding, 381*t*
- genomic PCR analysis, 382, 389*f*
- GFP fluorescence intensity variation, 387
- GFP fluorescent imaging, 382
- green fluorescent protein gene, 387*f*
- leaf disk bioassay, 383
- necrosis, 391*f*
- necrotic lesion development, 392*f*
- overview, 379
- Phytophthora megakarya, 385, 388*t*
- primer sequences, 383*t*
- semiquantitative RT-PCR, 382
- transformation vector construction, 381
- transgene expression analysis, 390*f*
- transgene insertion, 388
- transgenic cacao plant generation, 385
- transgenic lines generation, 381

Disulfide-bridged templated two-stranded peptide formation, 127
DNA, polymerase chain reaction, 454*f*
DNA blot analysis, thionin antifungal peptide synthesis, 374
Drosophila gut, 219

E

E. amylovora, 406, 408*f*
E. coli with Hth1, thionin antifungal peptide synthesis, 364, 366*f*
ELISA protocol, 113*f*, 121*f*, 125*f*, 129
Endostatin, 36*f*
- fragment, 29*f*

Erythrocyte lysis assay, 206
Expression, antifungal plant defensins, 318, 330
Expression vectors, thionin antifungal peptide synthesis, 367*f*

In Small Wonders: Peptides for Disease Control; Rajasekaran, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

F

Fire blight
  antibiotic resistance problem, 400
  causative agent, 398
  chemical control options, 399, 399*t*
    copper compound, 400
  economic importance, 398
  management, 398
Fluorescently labeled antifungal peptides, 346*f*
Four-cysteine, 272
Frog skin antimicrobial peptides
  alyteserin-1c, 54
  anti-infective agents, 49
  ascaphin-8, 54
  brevinin-1BYa, 56
  brevinin-1PRa, 56
  brevinin-2-related peptide, 52
  future clinical applications, 57
  kassinatuerin-1, 55
  molecular properties, 48
  natural, 51*t*
  nontoxic anti-infective agent
    transformation, 51*t*
  overview, 47
  pseudin-2, 55
  temporin-DRa, 50, 53*f*
Fungal pathogens, 302*t*
Fungal resistance, antifungal plant defensins, 330
Fungi interation, antifungal peptides, 342
Fusarium graminearum, HTH toxicity, 360, 362*f*

G

GCN4 stabilized HRC, 117
GCN4-HRC-GCN4, 117*f*, 118*f*
GCN4-HRC-GCN4 purification, 130
Gene cloning, thionin antifungal peptide synthesis, 372
Genomic DNA extraction, thionin antifungal peptide synthesis, 374
GENOPEP
  animal studies, 176
  assessment schedule, 179, 180*t*, 182*f*
  burn characteristics, 182
  burn mean percentage, 183*t*
  demography, 182, 182*t*
  design flow diagram, 178*f*
  drug description, 180*t*
  efficacy/tolerability variables, 178
  exclusion criteria, 181
  healing rate, 185*f*, 187*f*
  healing time, 185*f*
  inclusion criteria, 181
  overview, 173
  patients completely healed, 184*f*
  phase II/III study design, 176
  phase-I clinical studies, 176
  primary efficacy, 183
  primary tolerability, 183
  safety, 188
  secondary efficacy, 186
  secondary tolerability, 186
  statistical methods, 179
  subject deposition, 181, 181*t*
  testing procedures, 177
  in vitro antimicrobial studies, 176
  wound evaluation score, 179, 184*t*, 186*f*
  wound size, 183*f*
Glycine-rich cysteine-free, 273

H

HBD, 155
HDP. *See* host defence peptides (HDP)
Hevein-like peptides, 269
HIV, 143
Host defence peptides (HDP), 1
HRC, 102, 116, 120
HRC antibodies binding, S protein, 130
HRC antigenicity, 122
HRC immunogenicity, 109, 122
HRC immunogens, 105*f*
HRC peptides, 119*f*
HRC recognition, 124
HRC-GCN4, 117*f*, 118*f*
HRC-GCN4 purification, 130
HSV, 150
βHth, thionin antifungal peptide synthesis, 367
Hth cDNA cloning, thionin antifungal peptide synthesis, 362
HTH toxicity, thionin antifungal peptide synthesis, 361
Hyalophora cecropia, 80
Hydrophilic amino acids, 27*f*
Hydrophobic amino acids, 27*f*

I

IDR. *See* innate defence regulator (IDR) peptides
IgG purification, 129

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ix002

Iminopyrrolidine-containing peptides, 404, 405*f*
Immunization protocol, peptide immunogens, 128
Immunomodulatory cationic peptide therapeutics
  AMP, 2, 5
  broad spectrum antimicrobials, 6
  clinical development, 4*t*
  emerging technologies, 11
  HDP, 9*f*
  IDR, 2, 5
  overview, 1
  selective immunomodulatory agents, 8
  structures, 3*f*
  synthetic variants, 5
  vaccine adjuvants, 10
Inducible expression, plant AMP, 283
Inducible versus constitutive expression, AMP, 448
Inflammation, lytic peptides, 31
Influenza antigen microarrays
  antibody application, 197
  antibody labelling, 196
  detection, 198*f*, 199*f*
  fabrication, 196
  overview, 193
  protein expression, 195, 197*t*
  protein purification, 195, 196*f*
  working principles, 195*f*
Influenza virus, 150
Innate defence regulator (IDR) peptides, 2, 5
Intracellular killing, antifungal peptides, 348

K

Kassinatuerin-1, 55
Knottin-like family, 270
  cyclotides, 270

L

Leaf disk bioassay, Theobroma cacao, 383
Lemthio1, thionin antifungal peptide synthesis, 367, 370*f*, 373
Linear undecapeptides, 244*f*, 250*t*, 251*t*
Lipid transfer proteins (LTP), 268
Lytic peptides
  amino acid sequences, 34*t*
  amino-terminus, 26*f*
  angiogenesis, 28, 30*f*
  anticancer activity, 27
  anticancer therapeutics
    D2A21, 85*f*
    design principles, 80
    overview, 79
    systemic anticancer activity, 86
    tumor targeting, 87
    in vitro activity, 81
    in vivo activity, 86
  chemokines, 34
  endostatin, 36*f*
  endostatin fragment, 29*f*
  hydrophilic amino acids, 27*f*
  hydrophobic amino acids, 27*f*
  inflammation, 31
  osteoarthritis, 31, 32*f*
  overview, 21
  postulation, 37
  versus cytokines, 21

M

Magainins hybrids against *E. amylovora*, 410
Mammalian gut, 220
5′ methionine codon crowding effects, thionin antifungal peptide synthesis, 368
Microbial symbiont control, AMP, 216
Modes of action, antifungal plant defensins, 324

N

NCR peptide family, 223
NCR peptide interaction, 224
Nitrogen fixing endosymbionts, AMP
  bacterial differentiation, 227
  bacterial protection, 225
  NCR peptide family, 223
  NCR peptide interaction, 224
  symbiotic bacterial population establishment, 222
  symbiotic rhizobium bacteria differentiation, 222
Non-enveloped viruses, 152
Non-lytic modes, antifungal peptides, 342
Non-nuclear expression, transgenic plants, 306
  mitochondrial transformation, 307
  organelles, 308
  plasticid transformation, 307
  transgene expression, 307
  transgenic expression, 309

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ix002

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ix002

Nuclear expression, transgenic plants, 304

O

Oomycete pathogen resistance, antifungal plant defensins, 330
Organ-specific expression, AMP, 450
Osteoarthritis, 31, 32*f*
Oxypyrazole peptide
  antibacterial activity, 403*t*
  co-crystalisedorotic acid, hydrogen bonding interactions, 404*f*
  synthetic analogues, 404*f*

P

Pantocin, 408, 409*f*
Parainfluenza virus, 151
Pathogen killing, 255*f*
Pathogens, antifungal plant defensins, 320*t*
Peptide immunogens
  alpha helical conformation-specific antibodies development
    GCN4 stabilized HRC, 117
    HRC, 102, 116, 120
    HRC antigenicity, 122
    HRC immunogenicity, 109, 122
    HRC recognition, 124
    peptide-based vaccine, 103
    SARS-CoV infectivity neutralization, 111
    SARS-CoV S protein, 101
    synthesis, 103
    synthetic HRC, 108
    two-stranded coiled-coil template, 125
    two-stranded peptide immunogens, 110
    virus entry, 101
  circular dichroism spectroscopy, 129
  construction
    α-helical peptide epitopes stability, 99
    immunogenicity enhancement, 97
  disulfide-bridged templated two-stranded peptide formation, 127
  ELISA protocol, 129
  GCN4-HRC-GCN4 purification, 130
  HRC antibodies binding, S protein, 130
  HRC-GCN4 purification, 130
  IgG purification, 129
  immunization protocol, 128
  molar ellipticity, 105*t*
  overview, 93
  peptide scaffold-three-stranded HRC peptide formation, 128
  peptide synthesis, 127
  peptide-carrier protein conjugates preparation, 128
  SARS-S mediated syncytia formation, 130
  SARS-S pseudotyped retrovirus inhibition, 130
  synthetic peptide-based vaccines, 94
Peptide scaffold-three-stranded HRC immunogen, 109*f*
Peptide scaffold-three-stranded HRC peptide formation, 128
Peptide synthesis, 127
Peptide-based vaccine, 103
Peptide-carrier protein conjugates preparation, 128
pH-directed self-assembling helical peptide conformation
  CD spectra, 208*f*
  characterization, 205
  erythrocyte lysis assay, 206
  helical wheel structures, 207*f*
  modeling, 205
  overview, 203
  peptide sequences, 205*t*
  peptide synthesis, 205
  putative pore structure, 210*f*
Phytopathogens, AMP, 447
Phytophthora megakarya, 385, 388*t*
Phytotoxicity, AMP, 447
Piperidine-containing peptides, 404, 405*f*
Plant AMP
  β-barrelins, 272
  biological function, 273
  biotechnological applications, 274
  biotechnological approaches, 284
  defensins, 267
  four-cysteine, 272
  glycine-rich cysteine-free, 273
  hevein-like peptides, 269
  inducible expression, 283
  knottin-like family, 270
    cyclotides, 270
  LTP, 268
  overview, 265
  2S albumins, 272
  snakins, 269
  tertiary structure, 267*f*
  thionins, 266
  transgenic expression, 278, 279*f*, 280*t*, 282*t*
  transgenic plants, 276*t*
Plant disease control, AMP
  activity screening, 240

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ix002

  additional key properties screening, 243
  CECMEL11, 244, 247
  CYC10, 244, 247
  cyclic decapeptides, 244*f*
  flow diagram, 239*f*
  linear undecapeptides, 244*f*, 250*t*, 251*t*
  living organisms, 237
  overview, 235
  pathogen killing, 255*f*
  peptide properties optimization, 247*f*
  production costs, 254
  proof-of-concept, 252
  synthesis optimization, 254
  synthetic derivatives, 239
  toxicology, 253
Planta, thionins, 434*t*
Polyribosome analysis, thionin antifungal peptide synthesis, 374
Postulation, lytic peptides, 37
Potato plants
  pathogenic fungus, resistance, 452*f*
  Southern blot analyses of, 450*f*
Protein expression, 195, 197*t*
Protein purification, 195, 196*f*
Pseudin-2, 55
Putative pore structure, 210*f*
Pyrazole-conjugated peptides, 402

R

Representative thionin αHTH, tertiary structure, 423*f*
Rhizoctonia solani, 420*t*
  Membrane permeabilization of fungal phytopathogen, 421*f*
RNA analysis, thionin antifungal peptide synthesis, 374

S

2S albumins, 272
SARS-CoV, 151
SARS-CoV infectivity neutralization, 111
SARS-CoV S immunogen peptides, 112*f*
SARS-CoV S protein, 101
SARS-S mediated syncytia formation, 130
SARS-S protein-mediated syncytia formation, 116*f*
SARS-S pseudotyped retrovirus inhibition, 130
Seed- and leaf-specific thionins, 431*f*
Selective immunomodulatory agents, cationic peptides, 8
Snakins, 269
Streptomycin, 407*f*
Structure, antifungal plant defensins, 318
Structure-activity relationships, antifungal plant defensins, 324
Symbiotic bacterial population establishment, 222
Symbiotic rhizobium bacteria differentiation, 222
Synthetic HRC, 108
Synthetic peptide-based vaccines, 94
Synthetic variants, 5

T

Temporin-DRa, 50, 53*f*
Thionin, 266, 427*f*
  antimicrobial activity, 424
  crop disease resistance, 417
  endogenous activity
    antifungal protection, 432
    expression regulation, 429
    plant tissues, 430
  overview, 415
  plant defense system, 419
    allergenicity, 423
    antimicrobial activity, 420
    structure, 422
    toxicity, 423
  planta, 434*t*
  transgenic expression, 433, 434*t*
Thionin antifungal peptide synthesis, transgenic barley
  5′ methionine codon crowding effects, 368
  altered Hth1, 365
  cDNA library construction, 372
  cereal thionins, 360
  deduced amino acid sequences, 363*f*
  DNA blot analysis, 374
  E. coli with Hth1, 364, 366*f*
  expression vectors, 367*f*
  Fusarium graminearum, HTH toxicity, 360, 362*f*
  gene cloning, 372
  genomic DNA extraction, 374
  αHthΔ, 373
  αHth1, 373
  βHth, 367
  Hth cDNA cloning, 362
  αHthΔ mRNA translation analysis, 365*f*, 366, 368*f*
  HTH toxicity, 361
  Lemthio1, 367, 370*f*, 373

  - overview, 359
  - polyribosome analysis, 374
  - RNA analysis, 374
  - thionin toxicity plate assays, 371
  - thioredoxin-thionin fusion gene expression, 373
  - translation analysis, 371

Thionin toxicity plate assays, thionin antifungal peptide synthesis, 371

Thioredoxin-thionin fusion gene expression, 373

Transgene expression, 437*f*

Transgenic Arabidopsis lines, 437*f*

Transgenic crops, antifungal plant defensins, 330

Transgenic expression, plant AMP, 278, 279*f*, 280*t*, 282*t*

Transgenic plants, 276*t*

Translation analysis, thionin antifungal peptide synthesis, 371

Two-stranded coiled-coil template, 115*f*, 125

Two-stranded peptide immunogens, 110

Two-stranded peptide-conjugates, 108*f*

V

Vaccine adjuvants, cationic peptides, 10

Vaccinia virus, 151

Virus entry, alpha helical conformation-specific antibodies development, 101, 111*t*

W

Water channel, 427*f*

Downloaded by 89.163.35.42 on May 28, 2012 | http://pubs.acs.org
Publication Date (Web): April 4, 2012 | doi: 10.1021/bk-2012-1095.ix002